University of Alberta

Environmental biosafety of field scale plant molecular farming with safflower (*Carthamus tinctorius* L.)

by

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Abstract

The oilseed crop safflower (*Carthamus tinctorius* L.) has been transformed to produce high-value proteins for plant molecular farming (PMF) and resistance to a broad-spectrum herbicide. There are many potential benefits of PMF to society and industry. However, prior to commercialization of crops intended for PMF safety of the food/feed system and the environment must be assured. As part of a preliminary biosafety assessment I conducted a literature review and experiments to quantify potential exposure, gene flow, via pollen and seed from transgenic safflower to the environment and the food/feed system.

I evaluated the potential for pollen mediated gene flow (PMGF) from transgenic safflower to one or more of its wild/weedy relatives and to commodity safflower. Safflower is cross-compatible with several wild relatives in the Mediterranean, its center of origin. However, only two cross-compatible relatives occur in North America, *C. oxyacanthus* and *C. creticus*. PMGF from transgenic to commodity safflower was found to decline rapidly with distance from the pollen source. PMGF closest to the transgenic pollen source (0 to 3 m) ranged from 0.48 to 1.67% and rapidly declined to between 0.0024 to 0.03 % at distances of 50 to 100 m.

To quantify potential seed mediated gene flow (SMGF) from transgenic safflower, I examined seed losses at harvest, seed persistence in soil, efficacy of herbicides to control safflower volunteer survival and fecundity in follow crops. Total seed loss at harvest in commercial fields was large equivalent or well above the recommended seeding rate for safflower (150 viable seeds m^{-2}). Safflower has a relatively short longevity in the seed bank and viable seeds were not found in soil after two years.

Safflower volunteer plants in commercial fields and small plot experiments did not survive chemical fallow, but in some cereal and broad leaved crops a small number of plants did survive and generate small amounts of viable seed. Modified cultural practices (best management practices) could be adopted to mitigate PMGF and SMGF from transgenic safflower in the agroecosystem, but thresholds of zero are not practically or biologically realistic.

For Dad & Jaimie, and our loving memories of Mom.

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Chapter 1: Biosafety assessment of field scale plant molecular farming with safflower (*Carthamus tinctorius* L.) as a model crop

INTRODUCTION

Plant molecular farming (PMF) offers the capability to synthesize high-value pharmaceutical and bioindustrial products in plants at lower cost and, in some cases, superior quality than currently employed production systems (Goodman et al., 1987). After considerable research, an Alberta based company, SemBioSys Genetics Inc., has chosen safflower (*Carthamus tinctorius* L.) as a platform for PMF (Fig. 1-1). Constructs have been transferred into the nuclear genome of several safflower lines to enable seed-specific expression of high-value proteins and constitutive expression of resistance to the broad-spectrum herbicide glufosinate (L-*phosphinothricin*). Prior to commercial production of crops for PMF, they must be shown not to pose a significant risk to the environment and food/feed system. The Canadian Food and Inspection Agency (CFIA) regulate plants with novel traits (PNT) like herbicide resistance and PMF to ensure Canadians a safe environment, food and feed, and security of existing commodity markets. One of the requirements for regulatory decisions is an assessment of potential gene flow. Gene flow from a transgenic safflower may occur by pollen or seed through out its production cycle (transport, seeding to harvest and shipping). The consequences of unintended gene flow from regulated transgenic crops to food/feed crops can be severe.

Experiments were conducted to measure potential gene flow from transgenic safflower to the environment and the food/feed system.

DESCRIPTION OF SAFFLOWER

Safflower is a member of the Asteraceae (Compositae), subfamily Cynareae, thistle tribe Cardueae and is a part of the *Carthamus-Carduncellus* complex. Safflower has been grown in the Middle East and Northern Africa since 4500 BC (Dajue and Mundel, 1996). Historically, its seeds (achenes) were a source of food oil, and its florets were used as a source of yellow, orange, and red dyes (Carthamin and Carthamidin) for food and clothing (Howard et al., 1915; McGregor and Hay, 1952). The red-yellow and orange bindings of the Egyptian mummies were fabrics coloured with dyes extracted from safflower (Knowles, 1989; Weiss, 1983). After the commercialization of less expensive synthetic dyes (aniline), safflower has been grown predominantly for its seed oil (Knowles, 1989). Howard et al. (1915) noted that even after the synthetic dyes were on the market, the turbans of the Marwari traders of Rajputana were coloured with dye from safflower. More recently, battery powered backpack petal collectors have been developed for floret harvest after seed set to increase value of the crop in India (NARI, 2007).

Currently in the US, high oleic or high linoleic oil safflower types are grown for edible oil and the paint and varnish industry, respectively. After high-value oil is extracted from the seeds, the residue or seed meal is often fed to livestock, but is low in methionine and lysine. In Alberta, early varieties developed for the short growing season have lower oil content than US varieties, and are thus marketed for bird seed (Mundel et al., 2004). Safflower is currently a minor crop in Alberta with 320 to 810 ha grown

annually for bird seed (Mundel et al., 2004) and not used for human or animal consumption.

Subsequent thesis chapters detail relevant safflower characteristics, biology and ecology, as they relate to environmental biosafety. A brief description of safflower morphology and agronomy is, provided here as background. Safflower seeds are similar in shape to sunflower and similar in size to barley with a kernel weight of 0.03 to 0.05 g. It is an upright branching plant with spiny involucral bracts protecting each inflourescence (head). Cultivated safflower plants can be 30 to 150 cm in height, with a tap root that can penetrate the soil 2 to 3 m (Weiss, 1983). This deeply rooted crop improves soil drainage, and accesses moisture and nutrients at greater depths later in the growing season than most other crops, and grows best in well-drained sandy soils. Cool wet soils or heavy soils with poor drainage delay emergence and increase microbial infection of seeds and seedlings (Mundel et al., 2004). In Alberta, it can be grown under zero or reduced tillage management. Safflower is slow to bolt and develop a canopy to compete with weeds, and due to limited herbicide options, is best grown under low broad leaf weed pressure. This crop rarely lodges, is very resistant to wind and the heads do not shatter easily (McGregor and Hay, 1952; Mundel et al., 2004). It is relatively tolerant of high humidity and precipitation at early growth stages, and is susceptible to several leaf and head diseases (ie. *Botrytis* blight) after the plants bolt, and does best when atmospheric conditions are dry from flower bud formation to maturity (McGregor and Hay, 1952; Mundel et al., 2004). Extensive safflower crop losses in Manitoba in the early 1980s occurred due to the production of US varieties which were highly susceptible to early frost and disease (Mundel et al., 2004). Although, new varieties of safflower have

improved resistance to disease and earlier maturity, production remains limited to the southern region of Alberta because of drought tolerance and soil drainage requirements, response to disease, number of growing degree days and frost free days needed to reach maturity (Mundel et al., 2004).

HERBICIDE RESISTANCE

The transgenic safflower being developed for PMF is resistant to the herbicide glufosinate (L-*phosphinothricin*) which is a nonselective, broad-spectrum, post emergent, contact herbicide (Fig. 1-2) (Kudsk and Mathiassen, 2004; Vasil, 1996). It is sold as Liberty, Ignite or Rely in North America. This herbicide is classified as the lone member of group 10 based on its unique site of action (Mallory-Smith and Retzinger, 2003). Glufosinate (L-*phosphinothricin*) is a synthetic herbicide modified from an allelopathic chemical, *bialaphos* (L-*phosphinothricin*-L-alanyl-L-alanine), isolated from *Streptomyces hygroscopicus* (Fig. 1-2). Glufosinate inhibits the glutamine synthetase (GS) enzyme which is an enzyme involved in ammonium assimilation in plants (Fig. 1-3) (Nolte et al., 2004).

Ammonium assimilation in plants involves its conversion and assimilation to aspartate, carbamoyl phosphate, glutamate or glutamine (Fig. 1-2). There are two plant pathways to produce glutamate. The first involves the conversion of glutamate to glutamine catalyzed by glutamine synthetase (GS) and requires ATP (Fig. 1-2). Then glutamine oxoglutarate amidotransferase (GOGAT) catalyses the addition of an amide group to α–ketoglutarate to form two molecules of glutamate. A secondary means of ammonia assimilation occurs by the reactions catalyzed by glutamate dehydrogenase (GDH) and requires one NADPH (found in some bacteria and plants) (Nolte et al., 2004).

The primary mode of converting nitrogen to amino acids is through glutamate and glutamine. Thus, the enzyme GS has a critical role in plant maintenance, growth and survival.

Glufosinate irreversibly inhibits GS leading to rapid accumulation of ammonia generated from photorespiration and nitrate reduction, deficiency in several amino acids, and inhibition of photosynthesis and chloroplast structural disruption. The accumulation of ammonia and disruption of ribulose biphosphate carboxylase (RuBp) function leads to chloroplast dysfunction and plant death (Jansen et al., 2000; Maschhoff et al., 2000; Vasil, 1996).

Glufosinate consists of two enantiomers, D and L. The D-enantiomer is stable in plants and is not active against glutamine synthetase (GS) (Jansen et al., 2000). The L-enantiomer is an analog of glutamate and inhibits the synthesis of glutamine, which leads to a rapid interruption of nitrogen metabolism, accumulation of intracellular ammonia, the inhibition of RuBp carboxylase function in photorespiration and chloroplast distortion (Fig. 1-2) (Vasil, 1996). Glufosinate is highly active against its target enzyme (GS). It is not persistent in the environment because it is rapidly degraded by soil microbes (Vasil, 1996). Historically, glufosinate has been used for the maintenance of non-agricultural land. However, recent biotechnological developments have created glufosinate-resistant crops which increased the use and utility of this herbicide in agricultural settings.

Herbicide efficacy is largely influenced by several factors including time of application, uptake, translocation, and activity on the target site, plant species and growth stage and environment. Glufosinate is considered to have limited translocation in phloem and xylem and varied in the leaves of different species of grasses (Mersey et al., 2004;

Steckel et al., 1997). This limited uptake and translocation of glufosinate prevents efficient delivery to the target site (GS) and this accounts for reduced efficacy of this herbicide for some species.

Commercially grown crop plants resistant to foliar applied glufosinate were developed by transformation with one of two genes, *pat* and *bar* (different forms of phosphinothricin acetyltransferase), encoding the protein PAT to detoxify the herbicide. The genes were isolated from microbes (*Streptomyces viridochromogenses* and *Streptomyces hygroscopicus*) (that produce the herbicide (Droge-Laser et al., 1994; Maschhoff et al., 2000). The PAT enzyme catalyzes a reaction which leads to the inactivation of glufosinate by N-acetylation. Another gene, *gdhA*, has been isolated from *Escherichia coli*, which encodes a NADPH-dependent glutamine dehydrogenase (GDH) that increases ammonium assimilation. This is a novel mechanism for resistance to glufosinate. However, plants expressing *gdhA* were only resistant to six times the recommended application rates of glufosinate relative to non-transformed lines, whereas, plants expressing the *pat* or *bar* genes were 100 times more resistant than their non-transformed counterparts. It has been proposed that the *gdhA* gene could be used in conjunction with either the *bar* or *pat* genes to enhance glufosinate resistance in crops (Nolte et al., 2004). It has not been possible to generate plants resistant to glufosinate using mutagenesis. This difficulty and the low soil residual time of glufosinate suggest resistance to this herbicide by weeds has a low probability of occurring. However, the same arguments were once made for glyphosate resistance (Bradshaw et al., 1997).but weed species resistanct to glyphosate have been identified (Powles and Preston, 2006).

The CFIA requires data to determine if the products of crop plants with novel traits (PNT) crops are substantial equivalence to conventional products. Commercially grown glufosinate resistant crops include: *Zea mays* L. (maize), *Glycine max* L. (soybean), *Gossypium hirsutum* L. (cotton), *Oryza sativa* L. (rice), *Brassic napus* L. (oilseed rape), and *Beta vulgaris* L. (sugarbeet) (Nolte et al., 2004)). Several studies of transgenic plants resistant to glufosinate were found to be similar in agronomic performance to their non-transformed progenitors (tobacco; (*Nicotiana tabacum* L.) (De Greef et al., 1989; Mallory-Smith and Eberlein, 1996); potato (*Solanum nigrum* L.) (De Greef et al., 1989); and flax (*Linum usitatissimum* L.) (McHughen and Holm, 1995). In addition, glufosinate-tolerant *B. napus* (oilseed rape) was not found to be more invasive to rudural areas (disturbed by human activities) than non-herbicidal resistant plants (Crawley et al., 1993).

The use of glufosinate in forestry, orchards and non-cropped areas and now in agriculture with the innovations of herbicide-resistant crops has increased the utility of this herbicide. Its low soil persistence and the current lack of herbicide resistant weeds (Heap, 1997; Weed Science 2008) suggesting there is a low probability of glufosinate-resistant weed populations becoming a management concern in the near future. Herbicide-resistant crops rapidly metabolize glufosinate into non-toxic compounds and provide a relatively safe alternative to producers for weed control. Efforts should be made to prevent gene flow from glufosinate resistant crops to wild or weedy relatives as they could reduce the value of this herbicide in agriculture. However, hybrids of a crop and a weedy/wild relative or crop escapes in ruderal or natural areas are unlikely to be exposed to glufosinate. Thus, these crops are not likely to be more invasive than their non-transgenic counterparts.

PLANT MOLECULAR FARMING

From pre-agricultural times to today, humans have used plants as a source of food, animal feed, fibre, energy, construction materials, medicines, pleasure and religious rituals (Bosze and Balazs, 2000; Gleba et al., 1999; Kern, 2002). Recent advances in biotechnology allow the use of plants for manufacturing high-value proteins using genes introduced from different plant species or organisms (heterologous proteins) (Goodman et al., 1987). A Canadian based biotechnology company, SemBioSys Genetics Inc., has produced several transgenic lines of safflower with different constructs to produce seed-specific high-value proteins and resistance to the broad-spectrum herbicide glufosinate (Anonymous 2007). Plant molecular farming (PMF) is an innovative production system that may provide unique opportunities for the manufacture of high-value products in plants, including recombinant (heterologous) therapeutic (biologics) and diagnostic proteins (Gomord and Faye, 2004; Nykiforuk et al., 2006; Saalbach et al., 2001; Schunmann et al., 2002), enzymes for food/feed processing and industry, and feed stocks and specialty products for bio-industrial applications (Arai et al., 2004; Arcand and Arnison, 2004; Austin et al., 1994; Sarúul et al., 2002). Field production of PMF to produce high-value proteins like industrial enzymes and pharmaceuticals has many potential benefits to society. Significant progress has been made to engineer plants for the production of heterologous proteins, but many technical and safety challenges need to be addressed.

Potential value of PMF

The PMF industry intends to produce a diversity of proteins for pharmaceuticals, diagnostics, industrial polymers and enzymes, and nutraceuticals for humans and

animals. This technology may contribute to new innovations for industry to reduce environmental impacts by providing protein catalysts and building blocks to reduce energy requirements, environmental pollution and for biodegradable products. The global pharmaceutical sales for 2002 were estimated at US$430.3 billion representing a lucrative market (Larsen et al., 2007). It is predicted that novel systems to manufacture therapeutic proteins including PMF could reduce production costs by 15% and could yield profits for Canadian companies of up to US$1-2.5 billion by 2010 (Arcand and Arnison, 2004). However, regulatory compliance during production and segregating PMF seed from commodity seed after harvest and during transportation will be costly. For example, segregation for identity preservation of high erucic acid rapeseed is 15 to 25% of the cost of production (Smyth and Phillips, 2002). Thus, initially PMF will likely be restricted to the highest-value products.

The highest-value proteins being developed for PMF are intended for medical applications. The biotechnology industry has a capacity limitation to produce new pharmaceuticals (see alternative production systems, below) due to expensive infrastructure and time consuming construction (Garber, 2001; Gomord and Faye, 2004). In addition, protein based pharmaceuticals are being developed at a rapid rate and will be needed in large quantities in the near future including: monoclonal antibodies, recombinant blood proteins, plant-made oral vaccines and bio-defence products. Plant production platforms may increase capacity and decrease costs.

One high-value protein being produced in transgenic safflower is insulin which could aid in supply shortages predicted in the near future (Nykiforuk et al., 2006). This rise in demand stems from an aging population, increased obesity, an increased rate of diagnosis

and incidence of diabetes (Nykiforuk et al., 2006). In addition, Pfizer Inc. has recently gained approval in the US for a new insulin delivery system which uses an inhaler (pulmonary delivery) rather than parenteral (injection delivery) but requires five to twenty times the current dosage. Plant molecular farming may provide an option to increase capacity and reduce infrastructure costs relative to cell-based systems because scale-up of production can occur rapidly on an agricultural scale with existing knowledge and infrastructure (Arcand and Arnison, 2004; Boehm, 2006; Nykiforuk et al., 2006; Twyman et al., 2003).

While PMF is compelling for Canada, it is possibly more appealing for developing countries. For pharmaceuticals needed in developing countries, PMF may reduce cost and increased availability (Galeffi et al., 2005; Koprowski, 2005; Kumar et al., 2006; Rowlandson and Tackaberry, 2003; Sparrow et al., 2007). The cost of producing biologics (protein therapeutics) in plants is 15% lower relative to conventional pharmaceutical manufacturing (Arcand and Arnison, 2004). However, other authors have estimated the cost of recombinant protein production in plants relative to other host organisms to be 10 to 50 times lower (Giddings, 2001). Scale-up of transgenic plants intended for PMF can be more than 1000-fold per generation (Twyman et al., 2003). In addition, the infrastructure and knowledge for planting, growing and harvesting plants already exists in developing countries but may be lacking for large cell culture production. Compounds produced in seeds have been shown to remain stable for long periods of time and will not require refrigeration which will also decrease costs of infrastructure and storage. Thus, significant reductions in pharmaceutical costs and access to new protein based therapeutics could be realized by developing countries. Infectious

diseases account for 25% of deaths worldwide, and 45% of deaths in developing countries (Arntzen et al., 2005). The value of PMF to increase drug availability for developing nations is not just economical it has both social and ethical importance.

Potential products

Plant molecular farming may provide some options to industry and society for manufacturing raw materials or products and assist with resource management, environmental protection, and sustainable growth (Wilke, 1999). Plants are being developed to produce enzymes and other macromolecules that cannot be synthesized chemically, or can be manufactured in plants to reduced environmental impact and/or expense (Galeffi et al., 2005; Twyman et al., 2003). To date, the majority of cost-competitive agricultural products for industrial application has been limited to glucose, fuel-grade ethanol, organic acids, and bulk amino acids. Plant molecular farming may be conducted to produce specialized proteins with high-value including additives for manufacturing food and feed, biochemical catalysts (enzymes) for industry and chemical, polymers and polymerization, and biopharmaceuticals (recombinant protein based drugs, vaccines and antibodies) (Wilke, 1999).

A technology related to PMF involves transformed plants for phytoremediation which enables them to remove pollutants from the environment (Gleba et al., 1999). The expression of a single-chain antibody fragment (scFv) for the auxinic herbicide (group 4) picloram has been shown to protect tobacco from phytotoxicity and is planned for use in vegetation for remediation of contaminated soil (Almquist et al., 2004). The expression of bacterial nitroreductase in plants to breakdown TNT may provide a means to disarm land mines (Travis et al., 2007).

Increased interest in reducing environmental impacts from petroleum based products may increase the economic viability of biodegradable plastics. In addition, the replacement of glass fibres with plant fibres in plastic composites will increase the ability to compost bio-plastic products. Production of biodegradable plastics with bio-fibres (green bio-composites) will likely require transgenic technology research (Mohanty et al., 2002). The use of plant fibres has been shown to require 80% less energy than glass fibres (Mohanty et al., 2002). As well, biodegradable polymer based products to replace products like polyethylene plastic shopping bags are attractive for waste management and are produced from renewable resources (Riggle, 1998).

Plants may provide a way to reduce reliance on animals for production of some proteins. For example, the protein avidin has historically been extracted from eggs, but recent commercial production of this protein in corn seed has obtained yields of 20% total soluble protein. Thus, one bushel of maize expressing avidin at this level is equivalent to one tonne of eggs (Twyman et al., 2003).

Recently, the demand for protein-based medicines (biologics) is increasing due to new technologies, innovations and a large number of new therapeutic proteins have been approved for use (Walsh, 2003). Since the 1970s, protein-based pharmaceuticals have been produced by transgenic cells in large culturing facilities (Huot, 2003). In the US and Europe, 84 new biopharmaceuticals have been given approval and 500 more are under clinical evaluation. These recombinant proteins fall into eight product classes: blood factors, anticoagulants, hormones, hematopoietic growth factors, interferons and interleukins, vaccines, monoclonal antibodies, and several recombinant products in a new class (Ma et al., 2003; Walsh, 2003). These products include treatments for diabetes,

hemophilia, heart disease, various cancers, hepatitis, HIV, Alzheimer's disease, kidney disease, Crohn's disease, cystic fibrosis, multiple sclerosis, obesity autoimmunity, transplantation and arthritis (Anonymous, 2005; Galeffi et al., 2005; Walsh, 2003).

Currently, 25% of the prescription drugs in the US are derived from plants (Gleba et al., 1999). However, plant molecular farming may provide new alternatives for producing therapeutics. For example, the Pharma-Planta programme in the EU is developing topical applied anti-HIV monoclonal antibody (scFv) in maize and tobacco. The antibody produced successfully in CHO cells, has passed clinical trials (Sparrow et al., 2007).

The PMF industry wants to manufacture protein antigen based vaccines. Vaccines produced in plants have been shown to elicit the desired immune response in mice when delivered by injection, orally or to mucosal surfaces for Dengue virus (mosquito-borne pathogen), Newcastle disease virus, hepatitis B virus, foot and mouth disease virus (FMDV), measles, Norwalk virus, Classical swine fever virus (CSFV), pneumonic and bubonic plague (*Yersinia pestis*), *Taenia solium* (causative agent of cysticercosis in human and pig), *Escherichia coli* toxin (LT) B, *Bacillus anthracis* (causative agent of anthrax), (Alvarez et al., 2006; Bardor et al., 2003b; Chichester et al., 2007; DusSantos et al., 2005; Hernandez et al., 2007; Huang et al., 2005; Legocki et al., 2005; Ma et al., 2003; Moravec et al., 2007; Saejung et al., 2007; Tacket, 2005; Webster et al., 2005; Webster et al., 2006; Yang et al., 2007; Youm et al., 2007). In addition, a plant-made vaccine for plague (*Yersinia pestis*) has been shown to be efficacious in monkeys (Mett et al., 2007). Dow AgroSciences was given approval for the production and use of a poultry vaccine for Newcastle disease in tobacco cells growing in confined facilities in 2006. The vaccine was also shown to be efficacious in chickens when produced in rice (*Oryza*

sativa L.) (Wu et al., 2007). In the same year, a Cuban government agency (Cuba's Biotechnology and Genetic Engineering Centre; CIGB) was given approval for production and use of a monoclonal antibody produced in tobacco as part of a vaccine for hepatitis B for humans (Sparrow et al., 2007).

Alternative production systems

Several recombinant proteins are produced in bacterial species, such as *Escherichia coli*, which grow rapidly, are well characterized and are a relatively inexpensive to produce (Boehm, 2006; Gomord and Faye, 2004). Bacteria cells often do not modify proteins after translation and this can cause degradation or accumulation in inclusion bodies (Twyman et al., 2003). Thus, these systems are limited to simple proteins and peptides (Streatfield, 2007; Twyman et al., 2003). Therapeutic proteins requiring post-translational modification (PTM) are produced in eukaryotic systems using fungi (yeast), insect, mammal sterile cell cultures (mostly Chinese hamster ovary cells, CHO; mouse myeloma cell type NSO; baby hamster kidney, BHK; human embryonic kidney; HEK-293) or even transgenic animals (Boehm, 2006; Fischer et al., 2003; Gomord and Faye, 2004; Huot, 2003; Streatfield, 2007). These alternative production systems are expensive, limited by equipment scale up and on some occasions provide low yields, secretion problems, incorrect PTM and contamination with pathogens such as viral and animal protein(s) (Gomord and Faye, 2004; Ma et al., 2003; Streatfield, 2007). In addition, plant cells are able to produce, fold and assemble complex antibodies in a single cell line, whereas production in mammalian cells often require two cell lines (Twyman et al., 2003).

Plant production platforms

The production of more than 200 recombinant proteins in several plant species (platforms) has been demonstrated, but few have been commercialized (Fischer et al., 2003; Horn et al., 2004; Stoger et al., 2002a; Twyman et al., 2003). The industry has predominantly used tobacco (*Nicotiana tabacum* L.), rice (*Oryza sativa* L.) and corn (*Zea mays* L.) as production platforms (Boehm, 2006; Fischer et al., 2004). Other land plants being developed as platforms for PMF include soybean (*Glycine max* Merr.), common bean (*Phaseolus vulgaris* L.), safflower (*Carthamus tinctorius* L.), alfalfa (*Medicago sativa* L.), prairie carnation (*Saponaria vaccaria* L.), lettuce (*Lactuca sativa* L.), wheat (Triticum aestivum L.), barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.), tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.), banana (*Musa paradisiacal* L.), flax (*Linum usitatissimum* L.), cotton (*Gossypium hirsutum* L.), lupin (*Lupinus angustifolius* L.) and oilseed rape/canola (*Brassica napus* L.), (Austin et al., 1994; Boehm, 2006; Fischer et al., 2003; Fischer et al., 2004; Kumar et al., 2006; Perrin et al., 2000; Saruul et al., 2002; Schillberg et al., 2005; Schunmann et al., 2002; Stoger et al., 2002a; Twyman et al., 2003). Three aquatic angiosperms are being developed for contained PMF: duckweed (*Lemna minor* L.), *Spirodela oligorrhiza* Hegelm. and *Wolffia* sp. (Fischer et al., 2004). The Bryophyte (moss) *Physcomitrella patens* (Hedw.) Bruch & Schimp. is being developed for PMF (Decker and Reski, 2004; Fischer et al., 2004; Kumar et al., 2007; Twyman et al., 2003).The algal species *Chlamydomonas reinhardtii* and *Chlorella ellipsoidea* are being considered as molecular farming platforms (Boehm, 2006; Fischer et al., 2004; Franklin and Mayfield, 2004; Mayfield and Franklin, 2005).

ADVANTAGES, DISADVANTAGES AND FUTURE CHALLENGES FOR PMF

Optimization of heterologous protein yields in plants

Plant molecular farming has the potential to produce high-value therapeutic proteins on a larger scale at lower cost compared to other production systems (Gomord and Faye, 2004; Cramer et al., 1999). Economic viability of manufacturing proteins in plants will depend on the value of the protein and yields (Streatfield, 2007; Twyman et al., 2003). Yields will be primarily dependent on optimizated gene expression levels, accumulation of a stable form of the protein of interest and purification. Relative yields of recombinant protein will influence plant species and variety choices (Austin et al., 1994; Saruul et al., 2002; Sparrow et al., 2007; Streatfield, 2007; Tada et al., 2003).

Expression level optimization begins with designing genetic constructs for PMF (Schillberg et al., 2005). Transcription can be enhanced with global regulatory sequences added close to the promoter (Streatfield, 2007). Some plant introns have been shown to enhance expression when inserted into transgenes (Streatfield, 2007), which may contain gene regulatory sequence(s). Transcription levels can be enhanced with the addition of 5' non-translated regions including leader sequences from tobacco mosaic virus and potato virus X leader sequences. An appropriate consensus initiation site needs to be incorporated because these vary by plant species. In addition to high-levels of expression transcript stability can be enhanced by insertion of a polyadenylation target sequences after the stop codon. Potential constructs should be evaluated to ensure the mRNA produced does not form mRNA secondary structures and internal ribosomal binding sites that may prevent or limit translation (Streatfield, 2007). Similarly, prokaryotic sequences

from the construct vector and hairpin secondary structures at the DNA level or RNA double-strands after translation should be avoided (Twyman et al., 2003). In addition, when constructing transgenic proteins, amino acid sequences that trigger degradation or rapid turnover should be avoided.

Design of DNA constructs can incorporate sequence modifications to increase start codon recognition, codon usage bias and remove cryptic introns and instability sequences (Twyman et al., 2003). Transgene silencing, although not completely understood, is known to be epigenetic and to inhibit transgenes at the translational and post-transcriptional level (Twyman et al., 2003). Some precautions can be taken to prevent transgene silencing. These include incorporating the codon bias of the plant species used to manufacture a protein, which may differ from that of the species the gene was originally isolated. Altering codons (silent mutations) can increase expression, and prevent truncation, misincorporation and frameshifts (Ma et al., 2003; Streatfield, 2007). Some factors that can limit transgene expression are currently not controllable including the position of insertion, copy number, rearrangements and truncations. Multiple transformants are produced and screened to reduce these constraints in plants prior to field production. The development of a homologous recombination method would be invaluable to ensure transgenes are positioned in single copy form where they would have optimal expression (Ma et al., 2003).

The use of constitutive promoters can facilitate high transgenic proteins yields. The most common promoter used for eudicots is from the cauliflower mosaic virus (CaMV 35S) and for monocots the maize ubiquitin-1 (*ubi*-1) (Cramer et al., 1999; Streatfield, 2007; Twyman et al., 2003). In cereals, transcription can be elevated by the addition of an

intron in the 5' region of the construct (Ma et al., 2003; Twyman et al., 2003). A comparative study was conducted to examine the expression of the same single-chain Fv antibody in rice, wheat, pea and tobacco. The promoter used in the eudicot species, pea and tobacco, was the CaMV 35S and in the monocot species, rice and wheat, was the *ubi-1*. The highest transgenic protein yield per unit biomass was in rice but the highest overall yield was from tobacco because it generated the greatest biomass per unit area. The yield from wheat and pea seeds was the lowest (Twyman et al., 2003). Alternative constitutive promoters from plant viruses include cassava vein mosaic virus, C1 promotor from cotton leaf curl Multan virus and the promoter from component 8 of Milk vetch dwarf virus. A constitutive promoter isolated from *Agrobacterium* for mannopine synthetase has been shown to increase transcription relative to CaMV 35S. Leaf-specific constitutive promoters from ribulose-bisphosphate carboxylase have been used to produce over 1% total soluable transgenic protein (Twyman et al., 2003).

Signal sequences can be added to transgenic proteins to target specific subcellular (intracellular) compartments to increase accumulation and stability of the transgenic protein (Faye et al., 2005; Cramer et al., 1999; Ma et al., 2003). These include the cytosol, endoplasmic reticulum (ER), Golgi apparatus, apoplastic space, and the chloroplast (Boehm, 2006; Galeffi et al., 2005). Studies with tobacco have shown PTM are limited with cytosolic accumulations and protein yields are low. The lower yields in the cytosol may be due to its reducing environment and the presence of abundant proteases (Ma et al., 2003). An alternative to cytosolic targeting is the addition of a N-terminal signal sequence to direct protein to the secretory pathway (Ma et al., 2003). Proteins with only N-terminal signal directing them to the secretory pathway are

transported to the apoplastic space and may be excreted (Fischer et al., 2004; Ma et al., 2003). Protein concentrations are increased for proteins that are moved through the secretory pathway, and highest (2 to 10 fold) for those retained in the endoplasmic reticulum (Boehm, 2006; Ma et al., 2003; Schillberg et al., 2005; Twyman et al., 2003). Retention of proteins within the ER has been accomplished with C-terminal tetrapeptide sequences (HDEL or KDEL) (Cramer et al., 1999; Ma et al., 2003). The lumen of the ER enhances protein stability because it is an oxidizing environment, has few proteases, and several molecular chaperones (Ma et al., 2003).

The transformation of the chloroplast genome (transplastomic) rather than the nuclear genome (transgenic) can facilitate high accumulation in this organelle (6 to 46% total soluble protein) (Daniell et al., 2005; Ma et al., 2003; Streatfield, 2007). The chloroplast genome is attractive, because unlike the nuclear genome there is an absence of negative effects on transgene expression by positional effects and transgene silencing (Ma et al., 2003). In addition, the expression of polycistronic (multigene) constructs in tobacco chloroplast has been demonstrated for production of polyhydroxybutyrate (PHB), a biodegradable polyester, which requires three genes (Arai et al., 2004). Photosynthetic cells, which can contain thousands of chloroplasts, increase transgenic protein yield potential and are able to assemble complex proteins (Daniell et al., 2005; Ma et al., 2003). A large number of vaccine antigens and therapeutic proteins have been expressed in plants with transplastomic modifications (Arlen et al., 2007; Daniell et al., 2005). However, the types of proteins that can be manufactured by the chloroplast are limited due to an inability of this organelle to perform glycosylation (Ma et al., 2003) and successful chloroplast transformation has been limited to members of *Nicotiana*. One

biosafety advantage of integrating a transgene in the chloroplast genome rather than the nuclear genome is that this organelle is generally inherited maternally. This may reduce concerns of transgene contamination of neighbouring crops via pollen (Daniell, 2002). Although, this does not limit seed mediated gene flow. In addition, some workers have been concerned with the potential for horizontal transfer of the transgene from a plant chloroplast to bacteria, which has been verified under laboratory conditions (Ma et al., 2003; Twyman et al., 2003). However, it is not clear that this would occur in nature.

Alternatively, transgenic proteins can be tailored to have temporal protein expression with the timing dictated by a specific promoter (Ma et al., 2003). Protein accumulation in a specific tissue rather than the entire plant can limit exposure to the environment and facilitate protein stability. Inducible promoters have been used to express transgenes when a specific stimulus occurs (Cramer et al., 1999; Kumar et al., 2006; Ma et al., 2003; Streatfield, 2007). For example, a tobacco system has been developed where the transgene is driven by a peroxidase gene promoter which is active when the leaves are exposed to hydrogen peroxide, ultraviolet light, or sheared during harvest (wounding) (Fischer et al., 2004). The use of the peroxidase promoter increased protein yield 30% over the CAMV 35S (Fischer et al., 2004). Thus, the protein is not present until the plant biomass is harvested and within the processing facility (Cramer et al., 1999; Kumar et al., 2006; Ma et al., 2003). Other promoters have been investigated that can be induced by ethanol, dexamethasone and the insecticide methoxyfenozide (Jia et al., 2007; Ma et al., 2003; Sakvarelidze et al., 2007). This may provide a risk mitigation strategy because in absence of the stimuli, transgenic plants escaping containment to the environment would be less likely to produce the protein.

Transient expression systems have been developed to rapidly produce heterologous proteins without the incorporation of a genetic construct into a plant genome (Cramer et al., 1999; Kumar et al., 2007; Streatfield, 2007). This type of expression can be conducted by delivering a viral vector to a plant nucleus with agroinfiltration (vacuum infiltration to deliver *Agrobacterium tumefaciens* to plant tissue where the vector can enter the nucleus) (Fischer et al., 2004; Gils et al., 2005; Cramer et al., 1999; Kumar et al., 2007). These systems have been shown to produce high levels of transgenic protein that can be targeted with specific promoters to organs and subcellular compartments (Gils et al., 2005; Gleba et al., 2004; Cramer et al., 1999; Kumar et al., 2007). Viral systems can produce high amounts of protein but could have serious implications if released to the environment. Alternative systems using virulent viral vectors have been developed and shown to produce large amounts of transgenic protein in a short amount of time. A concern is that most viruses have wide host ranges and they cannot be used in open field situations (Schillberg et al., 2005). Recent research suggests some genetic use restriction technologies may improve the biosafety of viral production systems (Gleba et al., 2004). However, without strong assurance of environmental safety and segregation from crops these systems will require high levels of containment. Often transient expression systems have low capacity and without further refinement are probably best suited to testing constructs or performing experiments rather than commercial production (Kumar et al., 2007).

A disadvantage of plant leaves and other vegetative structures for heterologous protein accumulation is that they must be quickly transported and processed after harvest to prevent protein degradation. This limitation can be overcome by targeted protein

accumulation in storage organs such as seeds or tubers. Seeds have some advantages for long-term storage and often have specialized storage capacity (Ma et al., 2003). Seed-specific promoters have been isolated and characterized for monocots and eudicots and can be expressed in the embryo or endosperm (Deckers et al., 1999; Cramer et al., 1999; Moloney, 2000a; Moloney, 2000b; Perrin et al., 2000; Streatfield, 2007). Protein accumulation of heterologous proteins driven with seed-specific promoters for monocots have exceeded levels obtained with constitutive promoters (Streatfield, 2007).

Fusion proteins of a seed-specific protein with the transgene of interest have been used to target transgenic proteins to seeds. Initial attempts to couple recombinant proteins with seed storage proteins failed because post-translational cleavage limited their accumulation. Seed storage proteins called oleosins, are not cleaved after they are formed, and can make up 50% of an oilseed volume and in *B. napus* make up to 8-20% of the total seed protein (Cramer et al., 1999; Moloney, 2000a; Moloney, 2000b). Oleosins encapsulate oil-bodies and influence surface-to-volume ratio in oil-seeds of canola (*Brassica napus* L.), sunflower (*Helianthus annuus* L.), soybean (*Glycine max* L.), safflower (*Carthamus tinctorius* L.), flax (*Linum usitatissimum* L.), peanut (*Arachis hypogaea* L.), corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.). The transgenic oleosin protein coupled to a protein of interest can be extracted from seeds simply and inexpensively (Moloney, 2000a; Moloney, 2000b; Moloney, 2000b; Nykiforuk et al., 2006). Fusion of transgenic proteins with oleosin proteins have been shown to reduce purification costs and limit substances that can foul downstream processing apparatus (Deckers et al., 1999; Moloney, 2000a; Moloney, 2000b; Parmenter et al., 1995).

Other fusion protein systems have been developed. The fusion of a transgenic protein to the C-terminus of a single ubiquitin unit has been used to increase expression by 10-fold (Streatfield, 2007). Fusion of a transgenic protein with plant viral coat proteins can increase yield and simplify purification. In addition, the coat protein may be retained on plant-made vaccines to enhance immunogenicity (Streatfield, 2007). Affinity tags can be fused to the transgene and bound to a matrix during extraction, which may increase the stability of the protein and the amount of protein. (Faye et al., 2005; Cramer et al., 1999; Streatfield, 2007). After isolation, the affinity tag can be removed by proteolitic cleavage. One problem with affinity tags is that they may prevent appropriate folding or other protein modifications (Cramer et al., 1999).

Multiple proteins in one plant cell may be required for vaccines consisting of multiple antigens. The development of artificial chromosomes (Streatfield, 2007), tripartite genes (Walker and Vierstra, 2007), multiple transient infections to the same plant (Fischer et al., 2004) or transplastomic (chloroplast) (Arai et al., 2004) may facilitate the production of multiple proteins in one plant.

Modifications of heterologous proteins

Some recombinant therapeutic proteins require co- and post-translational modifications (PTM) to be biologically active for proper pharmacokinetics (i.e. clearance rate), stability and solubility (Faye et al., 2005; Gomord and Faye, 2004). Post-translational modifications of proteins that can be conducted by prokaryotes but are often not carried out include: proteolysis (cleavage of a signal peptide or cleavage of propeptide), formation of disulfide bridges (cross-linking), proper folding and oligomerization (association of peptides/proteins) (Gomord and Faye, 2004; Cramer et al., 1999).

Modifications prokaryotes cannot conduct involve the addition or modification of amino-acid residues via covalent bonding, such as the addition of one or more carbohydrates (glycosylation), fatty acids (S-acylation, N-myristoylation, prenylation, glypiation and cholesterol link), phosphate groups (phosphorylation), sulfates (sulfation), and/or vitamin-K-dependent reaction to form γ-carboxyglutamate (gamma-carboxylation), hydroxylation, stabilization by acetylation, isomerization/racemization (deamination), and carbonyl formation (oxidation) (Gomord and Faye, 2004; Cramer et al., 1999). Modifications can occur in the cytosol, endoplasmic reticulum, intra- and extracellular matrix, or various locations along the secretion pathway (Gomord and Faye, 2004; Cramer et al., 1999).

Transgenic plants have been used to produce complex proteins with appropriate PTM such as collagens, hemoglobins, immunoglobulins and secretory antibodies (Bardor et al., 2003b; Faye et al., 2005; Fischer et al., 2003; Gomord and Faye, 2004; Cramer et al., 1999; Ma et al., 2005; Moloney, 2000a; Moloney, 2000b) but for some proteins, the glycosylation (sugar) residues can differ from those produced by mammals. Mammal glycoproteins have a range of forms, but often have a core α (1, 6)-fructose with terminal galactose and sialic-acid residues. Plant glycosylation is identical to mammals until they are processed in the trans-Golgi apparatus (late stages) (Faye et al., 2005). At that time, plant glycoproteins contain the sugar moieties ß (1, 2)-xylose and α (1, 3)-fructose attached (Bardor et al., 2003b; Chen et al., 2005; Faye et al., 2005; Schahs et al., 2007; Schahs et al., 2007). The glycosylation of proteins can influence their physical and chemical properties such as thermal stability, resistance to proteolytic breakdown, and solubility which may influence their stability during production or storaege. These sugar

residues also influence biological function of proteins including immunogenicity, clearance rate and specificity of activity (ligand-receptor interaction) (Faye et al., 2005).

One concern of pharmaceuticals with plant glucans is they are known constituents of some plant allergens, and when administered to human patients, may cause an allergenic immune response (Chen et al., 2005; Gomord and Faye, 2004; Cramer et al., 1999). Thus, the administration of therapeutic proteins to humans or animals with these unique plant sugar residues may illicit an adverse immune response. Even if the immune response is not severe, it can accelerate the proteins clearance and decrease efficacy (Bardor et al., 2003a; Gomord and Faye, 2004; Cramer et al., 1999). A comparison of mammalian and plant vaccine for rabies (monoclonal antibody) demonstrated the plant form of the protein had a shorter half-life, but was equally efficacious to provide prophylactic protection against the rabies virus (Schahs et al., 2007). The rapid clearance of other non-human glycosylation residues on therapeutic proteins produced in yeast cell systems has been demonstrated (Gomord and Faye, 2004). Administration to mice of a recombinant antibody with plant-specific glycans did not elicit an immune response to these sugar residues (Twyman et al., 2003), but in another study rats and mice did produce antibodies specific to epitopes of plant N-glycans (Bardor et al., 2003a). Protein carbohydrate group eptitopes are rarely allergenic and plant glycoproteins are found in our diet (Chen et al., 2005; Ma et al., 2003). Interestingly, a large proportion of non-allergic human blood donors have antibodies specific to plant glycans (Bardor et al., 2003a; Chen et al., 2005). It is not clear how glycoprotein may improve or impede the efficacy and safety of pharmaceuticals and if there might be a difference between oral and injection delivery of biologics (Faye et al., 2005; Ma et al., 2003).

The alteration of the moss *Physcomitrella patens* to prevent fucosyltransferase and xylosyltransferase enzyme activity was recently demonstrated to prevent unwanted plant N-glycan addition to a transgenic protein. In addition, transfer of galactosyltransferase and sialyltransferase to produce proteins in plants with mammalian glycan terminal residues has been considered (Ma et al., 2003). This has been demonstrated in a tobacco system with successful addition of galactose residues, but failed to add sialic acid residues (Ma et al., 2003). A loss of function mutant of *Arabadopsis thaliana* has been used to produce a humanized monoclonal antibody identical glycosylation to the same protein produced in Chinese hamster ovarian cells (Schahs et al., 2007). There are several technical issues to overcome in order to use plants for mammalian protein production and these previous examples provide insight into why a developer may choose one plant species as a production platform over another.

Downstream processing of proteins from plant molecular farming

Up to 50 to 80% of the total cost of a biopharmaceutical can be associated with the downstream processing to obtain a purified and stable protein (Schillberg et al., 2005). Equipment currently employed to purify proteins from cell cultures need to be optimized or altered to remove plant specific substances like toxic alkaloid and phenolic compounds (Sparrow et al., 2007). Some methods have been devised to reduce or eliminate unwanted plant substances to improve downstream processing (Cramer et al., 1999).

Plant cell suspensions rather than whole plants may be used to limit the production of these substances, or proteins can be excreted via the secretory pathway to a hydroponics system (Ma et al., 2003; Sparrow et al., 2007; Streatfield, 2007). Suspension cells have been developed with tobacco, soybean, tomato, and rice. In addition, hairy root

disease caused by *Agrobacterium rhizogenes* has been used to produce recombinant proteins in tobacco suspension cells (Fischer et al., 2004; Stoger et al., 2002a). This eliminates the need to rupture cells to extract the protein and decreases downstream processing costs. However, cell suspensions and root secretion systems are best used in contained systems to facilitate protein recovery and avoid losses to the soil and groundwater. The use of plant sprouts for production rather than whole mature plants has shown to reduce problematic substances (Ma et al., 2003; Sparrow et al., 2007). Tobacco has been transformed with an antibody coupled to a transmembrane protein, which can be purified by extraction with buffers and detergents (Twyman et al., 2003). Expression of heterologous proteins in cereal seeds allows for highly concentrated protein in a small volume, reducing downstream processing costs (Twyman et al., 2003). As previously described, fusion proteins may simplify protein purification and reduce costs.

The oral or topical delivery of vaccines and drugs to patients or animals by consuming plant tissues expressing them is being developed (Arntzen et al., 2005; Rowlandson and Tackaberry, 2003; Streatfield, 2007). It is known that vaccination is the most efficacious means to prevent disease and World Health Organization (WHO) has identified a need for needle-free delivery of vaccines for 30 million unvaccinated children worldwide (Arntzen et al., 2005; Koprowski, 2005; Rowlandson and Tackaberry, 2003). Raw fruits and vegetables have been used to deliver recombinant subunit vaccines and topical application of semi-purified antibodies for passive immunization. Several vaccines are currently in clinical trials and have been shown to stimulate production of high levels of antibodies (Alvarez et al., 2006; Bardor et al., 2003b; Chichester et al., 2007; DusSantos et al., 2005; Hernandez et al., 2007; Huang et al., 2005; Legocki et al.,

2005; Ma et al., 2003; Moravec et al., 2007; Saejung et al., 2007; Tacket, 2005; Webster et al., 2005; Webster et al., 2006; Yang et al., 2007; Youm et al., 2007). Two significant problems remain with this strategy. First, the dosage or concentration of the pharmaceutical can be inconsistent among plant individuals because of variable environmental conditions during production or even among tubers, roots, leaves, fruits and seeds in the same individual plant (Sparrow et al., 2007). Second is the need for cooking some plant tissues or organs prior to consumption to increase palatability and digestibility, and to remove toxins, which often inactivates the pharmaceutical (Sparrow et al., 2007).

Further development of oral vaccines with PMF for the delivery of vaccines or drugs to wild animals that act as vectors for several diseases to humans and livestock could be very helpful (Martin-Alonso et al., 2003). Oral immunization of rabbits with potato tubers producing antigens for rabbit hemorrhagic disease virus has been shown to elicit the desire immune response (Martin-Alonso et al., 2003). The control of introduced possums (*Trichosurus vulpecula*) from Australia to New Zealand by immunocontraception has been developed in an edible carrot (*Daucus carota* L.) based vaccine (Polkinghorne et al., 2005). These vaccines might reduce costs and increase the number of animals treated. In addition, often these animals are not easily captured or capture is stressful to them. To date, oral vaccines for wild animals were not efficacious due to low concentration, stability, or presentation to the host immune system (Martin-Alonso et al., 2003). The environmental biosafety and regulation may be influenced by the production system and delivery method. One concern might be exposure of non-target organisms to oral vaccines and will require serious consideration.

Outlook for PMF

Examining the opportunities and constraints for PMF is still an area of very active research. Plants have several advantages to pharmaceutical production over other systems. Plants could provide a means to reduce the cost of production relative to other systems. They also have superior scalability through out the developmental phases from clinical I, II, III to production. They have reduced likelihood to harbour human pathogens. Researchers and industry have successfully used plants to produce, fold and modify, and assemble simple to complex multimeric proteins. The long term stable storage at ambient temperatures of transgenic proteins in plant seeds is attractive for pharmaceuticals that may be needed in large quantities when unpredictable epidemics or bioterrorism attacks occur. Plant systems provide a possibility for oral delivery of biologics from unprocessed or partially processed plant materials. The potential to reduce costs and accessibility of needed vaccines and medicines to people in developing countries could be invaluable. However, plant systems have several disadvantages that need to be considered or overcome. Currently, yields of proteins are not economically viable except for the highest-value proteins. There remains a long lag-time in development from gene to protein production relative to bacterial and yeast systems. Glycosylation of proteins needs to be consistent and in some cases may need significant modifications in the plant system or after purification. Further, the plant glycoforms influence on pharmaceutical safety and efficacy need careful study. Production of plants with pharmaceuticals requiring injection and without oral activity may be more tightly regulated by drug safety regulators (i. e. Health Canada or its equivalent in other countries), but may pose less of a concern to the environment. Conversly, therapeutics

produced in plants with the intent of oral delivery may pose more of an environmental concern and perhaps be less of a concern for drug safety (Sparrow et al., 2007). The focus of this thesis is the biosafety of the environmental and food/feed system from PMF. A balanced decision process is required to realize the benefits of PMF while ensuring safety to the public and environment.

Plant molecular farming may extend the benefits and use of plants from agriculture to society. A therapeutic molecule in a plant seed can be stored and remain stable at ambient temperatures for long periods of time (Ma et al., 2003). Antibodies have been shown to be stable without loss of activity for eight years in plant seeds (Sparrow et al., 2007). This may be beneficial for certain drugs/vaccines used for epidemics, bio-terror attacks or other unpredictable events (Stoger et al., 2002b). Plant molecular farming has the potential to contribute to diversification of our cropping systems and provide high-value crops for producers. It is envisioned that PMF will be conducted by a select few producers on limited acreage and could provide them with alternative income more lucrative than commodity crops, but would require changes to identity preservation and management practices.

Plant molecular farming may provide drugs and manufacturing capacity to the developed and developing world, reduce costs and increase therapeutic medicinal production globally, and potentially prevent contamination of drug supplies with animal pathogens (Sparrow et al., 2007; Streatfield, 2007).

The safety of PMF, especially of therapeutic proteins requires careful consideration. If plants expressing pharmaceuticals were consumed by animals in the environment or inadvertently by humans what would be the hazard? Both RNA and DNA molecules are

not inherently toxic and are consumed normally with food (Sparrow et al., 2007). Most proteins are not toxic and it is expected that if consumed, exposure would be low (Sparrow et al., 2007). However, one significant hurdle for the PMF industry is rigorous environmental testing and empirical data to evaluate the biosafety of production practices and a complimentary regulator framework.

BENEFITS OF TRANSGENIC PLANTS

In addition to the potential benefits of PMF, transgenic plants with production traits have been commercialized for over ten years and have contributed positively to producers and society. The commercial production of transgenic crops have provided several economic and environmental benefits (Beckie et al., 2006; Brookes and Barfoot, 2005; Brookes and Barfoot, 2006; James, 2006; James, 2003). The majority of transgenic crop acreage has been dedicated to herbicide, insect and viral resistant crops. In addition, growing transgenic crops has facilitated changes in pesticide products and their use, which has reduced the environmental footprint or load of agriculture (Brookes and Barfoot, 2006). Producers' adoption of transgenic crops has continually risen since their introduction in 1996 primarily driven by increased farm income. Herbicide and insect resistant crops have decreased fuel usage by reducing pesticide applications, and by increased adoption of reduce and zero tillage practices. In addition, reduce and zero tillage provides a net sequestration of carbon in soil, relative to a net loss by conventional tillage management. Not only have transgenic crops facilitated reductions in input cost, but have often increased yields and crop quality, which increase farm value (Beckie et al., 2006; Brookes and Barfoot, 2006). Transgenic crops have decreased health risks and increased benefits to producers and consumers. For example, insect resistant corn has been linked with a reduction in mycotoxins primarily from *Fusarium* spp. infections (Wu, 2007), which is a cause of throat cancer, liver problems and fetal defects in the developing world (Chassy et al., 2005). In addition, herbicide resistant canola, on average, has significantly reduced glucosinolates and chlorophyll content which can reduce nutritional quality and value of canola oil (Beckie et al., 2006). In addition, transgenic crops have reduced

production risk, increased producer convenience and reduced farm worker exposures to pesticides (Beckie et al., 2006; Brookes and Barfoot, 2006). Trangenic crops have altered the quantity and type of pesticides used, and the environmental impacts (Brookes and Barfoot, 2006)

Transgenic papaya (*Carica papaya* L.) resistant to the papaya ringspot virus in Hawaii is the Cinderella story for genetically modified foods. Papaya is the second largest fruit industry in Hawaii, and a ringspot virus epidemic threatened the future viability of the industry (Gonsalves and Ferreira, 2003; Gonsalves et al., 2004). Since the commercial production of transgenic papaya in Hawaii, total papaya acreage has increased and ringspot virus loads have been reduced, facilitating production of non-transgenic papaya. The technology was produced by a public sector scientist and has been given to the local farmers (Gonsalves and Ferreira, 2003; Gonsalves et al., 2004). In addition, the private sector (Syngenta) have developed and donated a genetically modified rice ("golden rice") enriched in vitamin A (ß-carotene) (http://www.goldenrice.org) in the hopes of improving nutrition and health of people in the developing world.

The acrage of transgenic crops grown is projected to increase in the near future (James, 2006). Biotechnology provides some opportunities to increase the amount of food, feed and fibre produced in the world and it has contributed to reductions in environmental impacts and negative impacts on farm workers. Plant molecular farming may provide new benefits to society, but the safety of this technology needs careful consideration by regulators and industry to ensure safety of the environment and food/feed system.

BIOSAFETY OF PLANTS WITH NOVEL TRAITS (PNT) IN CANADA

The Canadian Food Inspection Agency's (CFIA) Plant Biosafety Office (PBO) requires an environmental biosafety assessment for plants with novel traits (PNT) prior to field production, without confinement restrictions. The CFIA defines a PNT as "... a plant containing a trait not present in plants of the same species already existing as stable, cultivated populations in Canada, or is present at a level significantly outside the range of that trait in stable, cultivated populations of that plant species in Canada" (CFIA 2004). PNTs include those modified by mutagenesis, somaclonal variation, intra and inter-specific crosses, protoplast fusion, and recombinant DNA technology (transformation). In addition, PNTs includes plant species not previously grown in Canada and those with exemption are listed in Part V of the Seeds Regulations, crops grown out of containment prior to 1996. The CFIA has identified five key areas, "pillars", to be assessed for environmental safety which are: 1) the potential of the novel plant to become a weed of the agroecosystem or invasive to natural areas, 2) gene-flow to wild relatives, 3) potential for the novel plant to become a pest, 4) potential impact of novel plant or its gene products on non-target organisms, including humans, and 5) potential biodiversity impact(s) (CFIA 2004). My thesis research will contribute to environmental biosafety assessment for transgenic safflower.

The CFIA has provided detailed outlines for data requirements, but not specific protocols for developing data for its safety assessments for novel plants. They have released 70 cultivars with novel traits for cultivation or/and importation. The biotechnology industry prefers that CFIA designate precisely what data and analysis they

require for safety assessments of PNT, including those intended for PMF, ("bright lines") (CFIA 2007). The CFIA requests logical experimental designs and data collection with scientific rigor to enable decision making about potential risks. The CFIA is apprehensive to lay out strict predefined data requirements and often do not provide much direction in writing "faint lines and often voids" (no lines). One reason is the agency does not want to predefine the data requirements, because a new trait or crop may not require tests which were appropriate for previous evaluations. This open scheme also gives industry and CFIA flexibility to define new tests appropriate for new crop/trait(s) which may not have been conducted previously. Theoretically, this should allow flexibility, time and cost efficiencies to both the CFIA and industry. In addition, this framework should enhance harmonization with other government regulators and facilitate a smoother transition from commercial production to foreign markets (CFIA 2007) (personal communication, Dr. Philip Macdonald). However, this scheme can lead to gathering excessive and sometimes superfluous data for assessments which can add to the costs and uncertainty for both industry and regulators (Manalo and Ramon, 2007).

The CFIA has laid out well defined regulations for industry, "very bright lines", to follow for PNT developed with biotechnology intended for commercialization or importation into Canada. These include the determination if the plant is novel, how the PNT was developed, the novel gene and its products, the phenotype of the PNT and its conventional counterparts, and any known or potential environmental impacts expected if the PNT is growing. In order for the CFIA to determine the substantial equivalence of a PNT relative to its conventional counterpart to the five pillars used for the safety assessment, a detailed biology document is require for each species proposed (CFIA

2004). The biology documents contain information about the plant species, related species and potential for gene flow leading to introgression (movement of a gene from one species to another), geographical centre of origin(s), and interactions with other organisms in the environment. The CFIA recommends using the Organization for Economic Cooperation and Development (OECD) consensus documents as a guide (CFIA 2004). Biology documents currently exist for *Beta vulgaris* L. (sugar beet), *Brassica napus* L. (canola/rapeseed), *Brassica rapa* L. (canola/rapeseed), *Glycine max* (L.) Merr. (soybean), *Helianthus annuus* L. (sunflower), *Lens culinaris* Medikus (Lentil), *Linum usitatissimum* L. (flax), *Medicago sativa* L. (alfalfa), *Solanum tuberosum* L. (potato), *Triticum aestivum* L. (wheat), *Triticum turgidum* ssp. *durum* (durum wheat), and *Zea mays* L. (corn/maize) (CFIA 2004). The CFIA has a good record of safe release of PNTs in the Canadian agroecosystem and markets.

Although conventional agriculture is not risk free, and has significant consequences to the environment, plant molecular farming may pose additional biosafety concerns and the CFIA is cautiously developing a revised framework to evaluate environmental biosafety assessment of this new technology. To date, the CFIA have drafted a preliminary directive to assess the environmental safety of PNT intended for PMF (CFIA 2005) and an incomplete preliminary framework for regulating PMF (CFIA 2006). The CFIA is continuing to formulating a biosafety assessment framework through a transparent process with multiple stakeholders (CFIA 2001a, b). A significant hurdle to the completion of a regulatory framework for PMF has been identified by the CFIA as a socio-economic and ethics based policy outside of its mandate. This policy needs to be drafted by the Government of Canada (GoC) to gain guidance for acceptable risk of PMF

in Canada. These questions include: is PMF compatible with Canadian agriculture, should PMF be allowed in confined spaces or open fields, and should food/feed crops be used for PMF. The CFIA requires the GoC socio-economic policy and guidance on the benefits and acceptable risks of PMF, to set thresholds and develop regulations.

The CFIA is not the only government organization involved in the regulation of PMF. The CFIA is responsible for the environmental release of PNTs, the use of PNTS or their products in livestock feeds and veterinary biologics. These responsibilities overlap and in several cases are separate from those of other Canadian government departments. Health Canada is responsible for the regulation of pharmaceutical and biologics produced, sold and used in Canada. Environment Canada is responsible for the regulation of new industrial compounds; its mandate includes the protection of renewable resources including native species, and to enforce environmental policies and programs of the GoC. The Pest Management Regulatory Agency (PMRA) may be involved to assess the environmental safety of a plant with an altered phenotype if it is related to pesticides. It is not clear how these other departments and the CFIA will integrate policies and regulations for PMF (Norris, 2005; CFIA 2006).

The choice of plant species platform for PMF will have a significant impact on its regulation by Canadian government agencies, and potentially public perception. Food or feed crops have often been platforms for PMF because they often produce high yields, have refined cultural methods and are well characterized for transformation and protein expression. However, the CFIA recommends the plant platform have no, or limited use, for the food/feed. They recognize that a non-food/non-feed plant species may not be amenable to all PMF products or development (CFIA 2006). Several plant species may

not be appropriate for PMF because they are weeds or undomesticated species that would be difficult to contain in the environment (Sparrow et al., 2007). These species may not be amenable to genetic engineering or protein production (as previously discussed). The CFIA is concerned with the potential for admixture with food/feed commodities, but they do not suggest specific plant species for PMF platforms. Safflower may be suitable for PMF as it is grown as it is a non-food crop grown on low acreage in Canada.

The CFIA is open to the use of risk mitigation tools to limit gene flow from PMF via pollen or seed, to the environment and food/feed system. These include best management practices (BMP) and genetic use restriction technologies (GURTs). The BMP to mitigate gene flow from PMF activities have not been outlined specifically (CFIA 2006). A diversity of GURTs, genetic strategies to limit gene flow, have recently been reviewed (Hills et al., 2007; Mascia and Flavell, 2004; Murphy, 2007), but many of these would be considered novel and require an environmental biosafety assessment.

This thesis primarily addresses two of the five key biosafety areas established by the CFIA. These are the potential of safflower to become a weed of agroecosystems and potential for gene flow to wild/weedy relatives. Because of the concern for admixture of PMF safflower in food/feed crops and the absence of accepted thresholds, it describes temporal and spatial gene flow to quantify the sources of admixture. Further, it makes suggestions based on the results of the gene flow studies for best management practices to reduce admixture.

TRANSGENE MOVEMENT IN THE ENVIRONMENT AND FOOD/FEED SYSTEM

Regulated and unregulated transgenic plants have moved into the environment and have been detected in the food/feed system on several occasions mediated by pollen and seed. These situations have often been widely and sensationally reported and have altered the opinion and perception of biotechnology by the scientific community, public and government regulators. Developers of plant molecular farming will need to provide assurances that transgenic plants from commercial scale production will not move into the environment or the food/feed system above acceptable levels.

Pollen mediated gene flow is a significant concern for containing plants expressing transgenic proteins intended for PMF. It has been demonstrated that transgenes can move large distances via pollen in canola (*B. napus* L.); that herbicide resistant canola traits will be expressed, confer multiple herbicide resistance and that volunteers containing multiple traits can occur in fields in subsequent years. Hall et al. (2000) first documented novel herbicide resistance traits moved by pollen-mediated gene flow among three deregulated herbicide resistant canola varieties (PNTs) in a field in Alberta. Canola volunteers were reported to the authors by the producer, which had not been controlled by several applications of the herbicide glyphosate (N-phosphono methyl-glycine). Herbicide resistance in two of the varieties was conferred by transgenic constructs and the third by mutagenesis. Hall et al. (2000) investigation found canola volunteers with resistance to two and in some cases three herbicides (glufosinate, glyphosate and imazethapyr). In a follow up study, it was concluded that applications of relatively inexpensive herbicides (group 4) routinely used in cereal crops, controlled multiple

herbicide-resistant canola volunteers (Beckie et al., 2004). An extensive review of herbicide resistant crops and volunteer occurrence and control, has shown that most crop volunteerism has not increased and herbicides with alternative modes of action are often effective to control them (Beckie and Owen, 2007). Canada does not segregate GM/novel canola from conventional canola and practices have been adopted to limit the economic impact of volunteers in follow years.

Unregulated transgenic material in food or feed has lead to serious economic consequences. These instances have lead to changes in the regulatory policies and more stringent confinement procedures for field experiments. In addition, the biotechnology industry has responded with an increased awareness and diligence in safety compliance.

US regulators approval for feed but not food use of a transgenic corn, StarLink™ (Carter, 2004; [EPA] United States Environmental Protection Agency, 2007). This cultivar expressed a *Bacillus thuringiensis* gene (cry9C) confering resistant to insect pests and the construct was a minor variant of a previously approved cry9 protein. Some producers growing StarLink corn sold it to the more lucrative food market. The transgenic StarLink corn was admixed with other corn intended for the food market, and in September of 2000 the cry9C gene was detected in Taco Bell taco shells produced by Kraft Foods Inc. (Carter, 2004). At the time the safety of the protein for human consumption was unknown, and concerns over allergenicity triggered a recall of foods made from the StarLink corn, as well as imports of corn to Japan from the USA. The total costs of the recall, testing and lawsuits, were between $26 and 288 million US, and the StarLink corn developer, Aventis CropScience, losses are estimated in the billions (Arcand and Arnison, 2004; Schmitz et al., 2005). While subsequent studies conducted

showed the cry9C protein to be safe for human consumption, considerable negative public opinion and debate about safety of transgenic crops ensued (Carter, 2004; Vickner et al., 2003; United States Environmental Protection Agency [EPA], 2007). Internationally, regulators (especially in Canada and the US) responded with altered policies to make approvals for both food and feed (no-split approvals) prior to release from confinement and large-scale commercial production (Carter, 2004; Schmitz et al., 2005; CFIA 2001a).

The second instance occurred when confined field production of transgenic creeping bentgrass (*Agrostis stolonifera* L.) resistant to glyphosate (CP4 EPSPS), was shown to be insufficient to prevent pollen mediated gene flow to naturally occurring wild and weedy relatives (Watrud et al., 2004). The glyphosate resistant *A. stolonifera* was grown on *ca.* 162 hectares in a 4,453 ha isolation area in Oregon. Previous studies had shown natural hybrids can occur with six *Agrostis* spp. and some *Polypogon spp.*, and that pollen mediated gene flow could occur between *A. stolonifera* individuals up to 298 m (Watrud et al., 2004). Researchers positioned sentinel non-transgenic *A. stolonifera* plants around the isolated area in a circular fashion, from its edge up to 22 km away. In addition, they sampled resident *A. stolonifera* plants, other *Agrostis* spp. and *Polypogon monspeliensis* (L.) Desfontaines individuals naturally occurring in the same 22 km surrounding region. A large number of seeds harvested from the sentinel creeping *A. stolonifera* and resident *A. stolonifera* and *A. gigantea* Roth. (redtop bentgrass) plants were found to be transgenic. Using the outcrossing by distance data from (Watrud et al., 2004), models of wind trajectories (Van der Water et al., 2007) estimated that viable pollen may have been dispersed 75 km from the isolation area beyond the area sampled.

Containment of *Agrostis* spp. with glyphosate resistance gene (CP4 EPSPS) may prove to be difficult because of the competitive/weedy characteristics of these species, including vegetative reproduction by stolons, and seeds dispersed by animals, wind and water (Watrud et al., 2004). *Agrostis stolonifera* and *A. gigantea* are agrestral and ruderal weeds, and are found in natural habitats in the area. Further, transgene flow via pollen and seed may occur in subsequent years, and populations of *A. stolonifera* or *A. gigantea* with the CP4 EPSPS gene may become problematic weeds in agroecosystems if extensive glyphosate usage creates high selective pressure. Of particular concern would be reduced or zero tillage operations that are reliant on glyphosate for weed control, prior to seeding, rather than tillage or in turf and forest settings (Watrud et al., 2004). In a follow up study, it was shown that the movement of the glyphosate resistance gene had occurred into *A. stolonifera* populations, in non-agricultural areas 3.8 km from the "contained" isolation area, and had occurred by both pollen and seed mediated gene flow (Reichman et al., 2006). It is not clear if the frequency of the CP4 EPSPS gene will increase in natural populations of *A. stolonifera* or *A. gigantea*. Zhao et al. (2007) have shown hybrids produced between *A. stolonifera* and *A. gigantea* when the former is the male parent, have reduced pollen viability (29.9 ± 5.34 %), and backcrosses of these hybrids to *A. stolonifera* produced only a few nonviable seeds (Zhao et al., 2007).

Future field studies may include screening the Oregon endemic *A. capillaries* around the isolation area where transgenic *A. stolonifera* was produced, because hybrids among these two species have been shown to display hybrid vigour (Watrud et al., 2004). Hybrids of A. *stolonifera* and *A. capillaris* have reduced pollen viability (19.6 ± 6.95%)

when the former is the male parent. However, backcrosses of these hybrids with *A. stolonifera* produced seeds with a 75% germination rate (Zhao et al., 2007).

The third instance occurred when Prodigene Inc. conducted a small-scale field trial of corn expressing a seed specific antigen for a swine vaccine in 2001. Volunteer transgenic corn plants were sighted in the soybean follow crop in 2002, but were not removed prior to harvest. After harvest, vegetative material from the transgenic corn was suspected to have been commingled with the soybeans. The safety of the antigen to humans and animals if consumed, was not known at the time, and 500, 000 bushels of soybeans were ordered destroyed, and Prodigene was fined $2.8 million US (Arcand and Arnison, 2004; Elbehri, 2005; Pollack). The FDA later stated the corn material commingled with the large amount of soybeans posed little health risk (Arcand and Arnison, 2004; Elbehri, 2005).

The fourth instance occurred when pollen from a small plot experiment of transgenic corn intended for PMF of a pig vaccine, cross pollinated a field of corn intended for food /feed in Iowa. The neighbouring fields of 63 ha of corn growing near the site were ordered destroyed by burning. Prodigene was fined $250, 000 US and paid *ca.* $3 million US for follow up containment and clean-up (Elbehri, 2005; Ellstrand, 2003; Fox, 2003).

These events lead to an altered view of biotechnology by the public and government regulators. The response to PMF has remained cautious and not overly negative regarding PMF, likely because of the potential medical benefits (Kirk and McIntosh, 2005; Stewart and Knight, 2005). It also marked a significant turning point for the biotechnology industry because the government agencies responsible for the regulations of transgenic plants became more cautious (Fox, 2003; Stewart and Knight, 2005).

RISK ASSESSMENT

The evaluation of benefits and risk of PMF is still being formulated and debated. Risk is the likelihood or probability of a harmful event and can be thought of as a product of exposure and hazard (consequence). Risk assessment to the environment and food/feed system of PMF with safflower will be conducted by a science based process developed for other genetically modified plants (Garcia-Alonso et al., 2006; Johnson et al., 2007; Peterson and Arntzen, 2004; Raybould and Cooper, 2005; Raybould, 2006; Singh et al., 2006; Wolt and Peterson, 2000; Wolt et al., 2007). The assessment begins with the identification of what needs protection from harm, designated assessment endpoints. The endpoints will often be identified by policy and legislation, but can also be identified by producers (farms), researchers, the public and other stakeholders (Hails, 2002; Lomax, 2000; Meek and Keese, 2006; Peterson and Arntzen, 2004; Singh et al., 2006; Suter, 1990; von Krauss et al., 2004; CFIA 2001a, b). The next step is to development hypotheses regarding how PMF may cause harm to specific assessment endpoints. The third step is the use of pre-existing knowledge and, if necessary, new experimental data to test the hypotheses and quantify the potential harm to the assessment endpoints. The final step is the use of the information to determine the probability of risk. Government regulators can then use information about the benefits of a new technology and risk assessment to determine if the probability of risk is acceptable (Lomax, 2000; Raybould, 2006). However, benefits are not considered by CFIA. The risk assessment process begins and ends with input from stakeholders and scientific input between them (Raybould, 2006). Scientific research is a critical part of the process of risk assessment

but the public and government must be active participants for the process to be completed successfully (Peterson and Arntzen, 2004; Raybould, 2006).

The experiments in this thesis were designed to quantify the probability exposure of the environment and food/feed system from PMF with safflower. Problem formulation (identify harm) was developed by identifying the routes of possible exposure of transgenic safflower intended for PMF to the environment and food/feed system. The vector for exposure routes was considered to be pollen and seed because safflower is not propagated vegetatively. In addition, exposure routes were determined by considering the cultivation methods of safflower and its life cycle in an agroecosystem setting. Hypotheses were developed and tested with existing literature and gaps in knowledge needed to complete all of the risk assessment were determined. Experiments were designed to quantify aspects of transgenic and non-transgenic safflower biology and agronomy to quantify the potential for exposure to the environment and food/feed system to transgenic material. Exposure mitigation measures that may reduce risk of PMF with safflower by modification of production management practices have been suggested after each risk assessment. The acceptable risk of PMF with safflower in the Canadian agroecosystems will require designation of acceptable risk and risk assessment by the CFIA. The work presented here will aid in the risk assessment process by providing the potential routes and amount of exposure of transgenic safflower to the environment and food/feed system.

EXPERIMENTAL QUESTIONS

Using safflower as a model system, I attempted to address key questions on the environmental biosafety and ability to segregate a crop containing a PMF. Initially, I

addressed two of the five key areas or pillars set out by the CFIA and prioritized concerns specific for safflower. Three of these pillars are primarily associated with the specific protein expressed for PMF and were not addressed in my thesis work. Two of these pillars include concerns of pollen mediated gene flow to wild/weedy relatives and pollen and seed mediated gene flow from transgenic to commodity safflower. I also identified additional concerns of seed mediated gene flow. Seed mediated gene flow may occur by inadvertent mixing (admixture) of transgenic safflower intended for PMF with commodity crops or the environment may occur by several means. In addition, safflower seed lost at harvest could persist in the environment and volunteer in subsequent growing seasons facilitating gene flow to the environment or food/feed system via pollen or seed. The quantification of safflower seed and pollen mediated gene flow may aid in development of mitigation procedures to limit these risks.

Pollen mediated gene flow

Crop species and varieties have different rates of outcrossing and autogamy that are controlled genetically but are influenced by the environment (Eastham and Sweet, 2002). Pollen dispersal, for both wind and insect pollinated species, is greatest amont nearest neighbours and declines with distance. Major factors affecting outcrossing among populations include: the number of plant species attractive to pollinators in the area, isolation distance, form and density of donor and receptor plant populations, geographic and vegetative barriers, wind direction and speed, floral synchrony, floral position on the plant, ploidy level of all populations /species concerned, genetic compatibility and pollen longevity (Luna et al., 2001; Rognli et al., 2000). Management that incorporates spatial and temporal isolation has been used by seed growers to maintain varietal purity and

could minimize gene flow between crops and volunteer populations. Vegetative barriers may reduce pollen movement but some long distance dispersal may still occur. Genetic barriers or genetic use restriction technologies (GURTs) could be a means of reducing gene flow via seed and pollen but these systems have not as yet been opperationalized and have additional concerns. These novel gene containment strategies will likely be considered novel in Canada and require a separate evaluated for environmental risk.

Pollen mediated gene flow from transgenic safflower to a wild relative

Most crops species are cross-compatible with one or more with wild/weedy relatives (Messeguer, 2003). Hybridization and subsequent introgression have been documented between crops and their wild relatives (Messeguer, 2003). Hybridization is the first step to the stable incorporation of a gene for set of genes into a population. Factors determining the establishment and persistence of a trangene in a wild population including: floral synchrony and spatial sympatry, hybrid fitness, compatibility, mode of pollination, seed dispersal mechanism(s), and others (Messeguer, 2003 as reviewed in Ellstrand 1999 and Hancock et al 1996). Introgression has lead to the development of hybrid populations that have become weedier than their original parents (reviewed by Ellstrand 1999). The ecological fitness or cost of a trangene needs to be considered to determine if the wild plant will become invasive or weedy.

In order for transgenes to move from PMF safflower to a wild relative, they must be both geographically sympatric and cross-compatible. In chapters 2 and 3 I assess the potential for transgenic safflower to hybridization with wild relatives and establishment of the transgenes in these populations (introgression). The geographic distribution of wild safflower relatives and their cross-compatibility with safflower are reviewed in Chapter

2. These data were used to identify areas where cultivation of transgenic safflower for PMF should be avoided. The domestication of safflower and the weedy/wild characteristics of its wild relatives are reviewed in Chapter 3. This review was conducted to compare cultivated safflower to its wild relatives to provide hypothesis of how likely domestic safflower is to become a weed in the future.

Pollen-mediated gene flow from transgenic to commodity safflower

To mitigate the risk of transgene movement via pollen from PMF to commodity safflower, an appropriate isolation distance must be established. To determine this distance, several field experiments in diverse environments were conducted using transgenic plants (pollen source) neighboured by non-transgenic plants (pollen trap). In Chapter 4, I assess the potential for pollen mediated gene flow from PMF safflower to a commodity safflower intended for the food/feed system. Previous studies have shown safflower outcrossing to be primarily mediated by insects but wind also moved pollen among plants 122 cm apart. Different cultivated varieties evaluated in the same field ranged in outcrossing rates from 0 to 100% which suggests this characteristic is influenced by genotype (Claassen, 1950). The outcrossing rate of transgenic 'Centennial' safflower intended for PMF was unknown.

Seed mediated gene flow

Seed mediated gene flow could occur via several avenues during or after production of transgenic safflower for PMF. I present results of several field and greenhouse experiments and surveys of commercial safflower fields to quantify seed mediated gene flow from transgenic safflower to the environment and food/feed system is in Chapters 5 and 6. Volunteer PMF safflower in following years could arise from seeds lost at harvest.

These volunteers could extend the potential for gene flow via pollen to commodity safflower (Hall et al., 2000) or/and their seed could lead to admixtures in future years. The survivorship and fecundity (seed production) of volunteer PMF safflower was quantified in different crops including both HT and commodity canola and cereal crops. A comparison of PMF and commodity safflower persistence in the seed bank was compared and assessed. In addition, a survey of commercial fields where safflower had been grown in the previous year was conducted to quantify post-harvest volunteerism under normal production practices.

CONCLUSIONS

My research examined biosafety and risk mitigation of PMF safflower. Baseline data are required for safflower to evaluate how transgenes influence its potential to become weedy, interact with the agroecosystem relative to commodity safflower, and how seed and pollen from transgenic safflower might enter the environment or the food / feed systems. Initial literature reviews were conducted to determine gaps in our knowledge and design studies to gain an understanding of safflower biology in relation to the biosafety of this new technology using this crop as a platform. Studies presented here address several aspects of safflower biology and cultivation in relation to biosafety. This research provides industry and government regulators with data relevant to safety assessment and best management practices to mitigate risks associated with PMF safflower.

Figure 1-1. Two safflower inflorescences surrounded by spiny bracts.

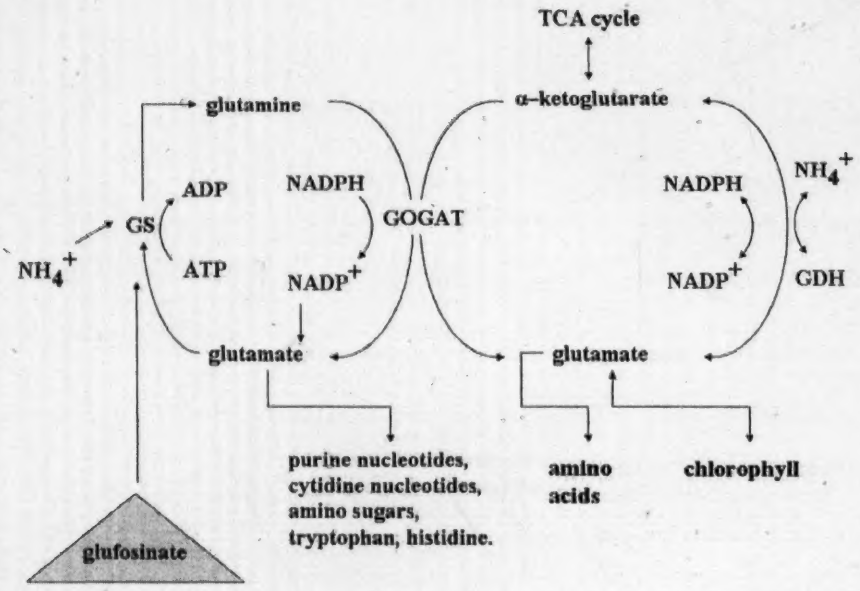

Figure 1-2. A schematic drawing depicting the influence of glufosinate on nitrogen metabolism in plants. Inhibition of glutamine synthetase (GS) leads to rapid accumulation of ammonia generated from photorespiration and nitrate reduction, deficiency in several amino acids, inhibition of photosynthesis and chloroplast structural disruption (Jansen et al., 2000; Maschhoff et al., 2000) Redrawn from Nolte et al. (2004) and Mathews and van Holde (1995). Note NH^{4+} occurs at physiological pH, but the unshared e- pair from NH_3 is the actual reactive species.

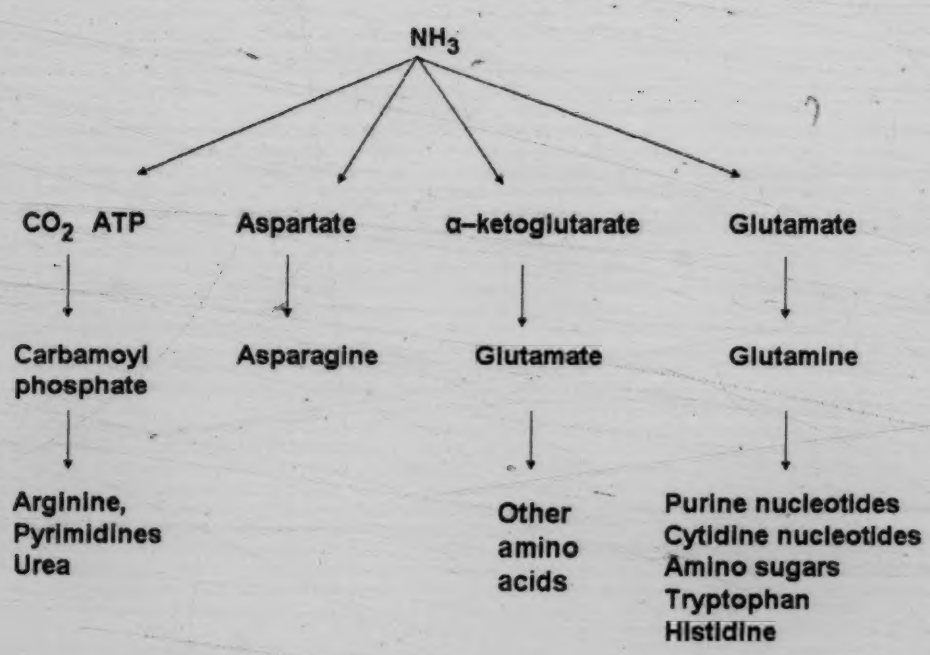

Figure 1-3. Nitrate as precursor in plants. Redrawn from Mathews and van Holde (1995).

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Chapter 2: Theoretical hybridization potential of transgenic safflower (*Carthamus tinctorius* L.) with weedy relatives in the New World

From: McPherson MA, Good AG, Topinka AKC, Hall LM (2004) Theoretical hybridization potential of transgenic safflower (*Carthamus tinctorius* L.) with weedy relatives in the New World. *Can. J. Plant Sci.* 84: 923-934.

Carthamus tinctorius L. (safflower) is being evaluated as a crop for the production of plant-made pharmaceuticals using an oleosin fusion protein system. I evaluated the potential for transgenic gene flow from *C. tinctorius* to wild or weedy relatives in Western Canada. Cytogenetic and phylogenetic studies with most of the species of *Carthamus* have demonstrated that cultivated *C. tinctorius* has the ability to hybridize with at least six wild or weedy relatives worldwide. Of the four naturalized wild relatives in the New World, only two, *C. oxyacanthus* and *C. creticus*, have successfully been crossed with *C. tinctorius* to produce fertile hybrids. Data from artificial crosses resulting in fertile offspring indicate the biological potential of a hybridization event, but only if the species flower synchronously and are sympatric can this occur. Based on the New World distribution of *C. oxyacanthus* and *C. creticus* I predict that hybridization with transgenic *C. tinctorius* could occur in some areas of Argentina, Chile Canada (Alberta and British Columbia) and localities within several states in the USA including Arizona, California, Coloradoa, Idaho, Florida, Illinois, Iowa, Kansas, Massachusetts, Montana, Nebraska, New Mexico, North Dakota, Ohio, Oklahoma, Oregon, Utah, Texas and Washington. Locations in the New World where wild species of *Carthamus* have not

been naturalized may provide biologically isolated locations for the cultivation of a transgenic safflower crop.

Keywords: *Carthamus*, safflower, transgenic, hybrid, gene flow, introgression.

McPherson, M. A., Good, A. G., Topinka, A. K. C. et Hall, L. M. 2004. Potentiel d'hybridation théorique du carthame trans-génique (*Carthamus tinctorius* L.) avec les adventices apparentées d'Amérique. *Can. J. Plant Sci.* 84: 923-934.

On s'intéresse au carthame (*Carthamus tinctorius* L.) pour la production de substances pharmaceutiques végétales par le biais d'un système de protéines hybrides de l'oléosine. Les auteurs ont tenté d'évaluer les risques de flux génétique entre le carthame et les espèces sauvages ou adventices apparentées qu'on trouve dans l'ouest du Canada. Les études cytogénétiques et phylogénétiques sur la majorité des espèces du genre *Carthamus* indiquent que le carthame pourrait se reproduire avec au moins six espèces sauvages ou adventices dans le monde. Sur les quatre qui se sont acclimatées en Amérique, seules *C. oxyacanthus* et *C. creticus* ont donné des hybrides fertiles après croisement avec *C. tinctorius*. Les données sur les croisements qui ont abouti à des hybrides fertiles nous renseignent sur le potentiel biologique d'hybridation, mais un croisement ne peut survenir que si les deux espèces sont sympa-triques dans le temps et l'espace. Compte tenu de l'aire de distribution de *C. oxyacanthus* et de *C. creticus* en Amérique, les auteurs estiment qu'il pourrait y avoir hybridation dans certaines parties de l'Argentine, du Chili et de plusieurs États américains, dont la Californie, la Floride, l'Illinois, le Kansas, le Nouveau-Mexique, l'Ohio, l'Oklahoma, l'Orégon, l'Utah et le Texas. Les endroits d'Amérique où les espèces sauvages du genre *Carthamus* ne se sont pas acclimatées

pourraient constituer des endroits biologique-ment isolés où l'on pourrait cultiver le carthame transgénique.

Mots clés: *Carthamus*, carthame, transgénique, hybride, flux génétique, introgression

INTRODUCTION

Safflower (*Carthamus tinctorius* L.), one of humanities' oldest crops (Johnston et al., 2002), is currently being evaluated as a host for a new transgenic technology, plant-made protein-based pharmaceuticals (Lacey et al., 1998). Safflower was originally grown to produce dyes (carthamine) for food and fabric, and for medicinal use; but is currently cultivated for edible oil and birdseed. Annual world production of safflower is estimated at 800,000 t, (Gyulai, 1996) not including a large number of small garden plots throughout India and Pakistan harvested for local use (Johnston et al., 2002). Safflower is thought to have originated in the Euphrates basin (Knowles, 1969; 1989) and from this center of origin, cultivation expanded to Egypt, Ethiopia, southern Europe, and southern Asia and the Far East (Smith, 1996). Hybridization with several wild species of *Carthamus* may have played a major role in the evolution of *C. tinctorius* in the Mediterranean and Asia where they are sympatric (Ashri and Knowles, 1960; Schank and Knowles, 1964). Cultivation of safflower in the New World began in 1899, and it was commercially grown in the 1950's (Knowles, 1958; 1989). Weedy and wild relatives of C. *tinctorius* were naturalized in the New World as early as 1891 (Fuller, 1979).

A cultivar of *C. tinctorius* has been genetically engineered to express two novel traits. The first is a nuclear encoded gene cassette conferring herbicide resistance. The second entailes the fusion of a gene encoding a plant made pharmaceuticals with the pre-existing gene encoding the seed protein oleosin. The utility of an oleosin fusion protein to

facilitate the production of a protein of interest in an oilseed has been described previously (Parmenter et al., 1995) and the structure and isolation of the oleosin protein in safflower demonstrated by Lacey et al. (1998). The environmental biosafety implications of plant-made pharmaceuticals production in safflower are currently being evaluated.

Gene escape from transgenic crops to wild or weedy relatives is a significant environmental safety concern. Hybridization is the first requisite step for gene escape (Rieseberg and Ellstrand, 1993; Rieseberg and Wendel, 1993). Species must flower synchronously and be close enough spatially (sympatric) for pollen mediated gene flow to occur in order for hybridization to occur. Introgression, the stable incorporation of a gene into a wild or weedy population, can occur if a crop / wild species hybrid successfully backcrosses to individuals of either species (Conner et al., 2003; Ellstrand et al., 1999). Hybridization and introgression between crops and their wild relatives is common and has been documented for 12 of 13 different crop species evaluated by Ellstrand et al. (1999). For example, introgression has been studied in *Oryza sativa* L. (rice; Lu et al., 2002), *Helianthus annuus* L. (sunflower; Linder et al., 1998), *Raphanus sativus* L. (cultivated radish; Snow et al., 2001), and *Phaseolus vulgaris* L. (common bean; Beebe et al., 1997). Transgenic movement to weedy or wild relatives has been documented from *Brassica napus* L. (oilseed rape; Metz et al., 1997).

Hybridization with *C. tinctorius* and several wild relatives has been demonstrated to occur artificially (hand pollination; Ashri and Efron, 1964; Ashri and Knowles, 1960; Claassen, 1950; Estilai, 1977; Estilai and Knowles, 1976, 1978; Heaton and Klisiewicz, 1981; Imrie and Knowles, 1970; Kadam and Patankar, 1942; Khidir and Knowles, 1970a,

b) and naturally (open pollination; Ashri and Rudich, 1965; Claassen, 1950; Kadam and Patankar, 1942) However, cross-compatibility of species, while relatively easy to measure, is not by itself, a sufficient predictor of gene flow potential under field conditions. The cultivation of safflower and naturalization of several wild relatives in the New World suggests there is a potential for hybridization between these species in areas of co-occurrence.

To assess the potential for gene escape through introgression from cultivated *Carthamus tinctorius* (safflower) to wild relatives, the phylogenetic relationships in *Carthamus* were reviewed. Closely related species are presumably more likely to hybridize successfully, but this inference must be substantiated by empirical data derived from both open and artificial crosses. The primary source of data used to infer phylogenetic relationships in the genus *Carthamus* has been inferred from artificial and natural interspecific hybridization experiments. Relationships have been inferred from the likelihood of obtaining viable F_1 hybrids and observations of chromosome behavior (normal/abnormal) when these hybrids occurred. The geographic distribution and biology of New World *Carthamus* species with the potential to hybridize with cultivated safflower were assessed to determine documented spatial sympatry and temporal floral synchrony. Finally, I highlight directions for future research to verify the potential for interspecific gene flow from a transgenic safflower crop in North and South America.

RESULTS FROM THE LITERATURE

Taxonomy and phylogeny

Carthamus L. is a genus of *ca.* 16 species *sensu* López-González (1989) (Table 1). It is a member of the subtribe Centaureinae, tribe Cardueae (thistles), subfamily Tubuliflorae and family Asteraceae (Compositae) (Kumar, 1991; Vilatersana et al., 2000). The position of *Carthamus* in the tribe Cardueae is unclear, as is the circumscription of the genus (Vilatersana et al., 2000).

The taxonomy and classification of *Carthamus* has changed substantially as data for this diverse group has been obtained and interpreted (Table 1). To synthesize the distribution, hybridization and cytological literature for *Carthamus*, it was necessary to decipher and compare the different taxonomic schemes used by researchers over time (Table 1). For clarity, I follow the classification scheme of López-González (1989) unless otherwise stated. This classification system is based on information from morphology, biogeography, cytology and interspecific compatibility.

Delimitation of *Carthamus* and a close ally, *Carduncellus*, has been difficult due to morphological similarities and convergent evolution of several variable characters utilized by taxonomists (Vilatersana et al., 2000). These two genera constitute a large group termed the *Carduncellus-Carthamus* complex (Vilatersana et al., 2000). Morphological and cytological characters identified to date are insufficient to delimit the species of the *Carduncellus-Carthamus* complex into discrete sections and genera (Dittrich, 1969; Hanelt, 1963; Lopez-Gonzalez, 1989; Vilatersana et al., 2000). Depending on the taxonomist and the morphological characters emphasized in their classification scheme some species in the complex have been moved in and out of

Carthamus and *Carduncellus* (Table 1) (Vilatersana et al., 2000). A new genus *Femeniasia* Susanna was recently added to the complex, and has further complicated our understanding of relationships among these closely related groups (Vilatersana et al., 2000). A recent molecular based phylogenetic study (Vilatersana et al., 2000), several detailed morphological studies (Ashri and Knowles, 1960; Hanelt, 1963; Lopez-Gonzalez, 1989) and cytogenetic analysis of hybrids from interspecific crosses (Ashri and Knowles, 1960; Estilai and Knowles, 1976, 1978) have been used to transfer several closely related thistle species from *Carthamus* to two other genera (Table 1; *Lamottea* and *Phonus*). Five chromosome groups were identified by Ashri and Knowles (1960) in *Carthamus* (n = 10, 11, 12, 22 and 32) and these have influenced the delimitation of the sections of the genus (Table 1).

Vilatersana et al. (2000) analyzed DNA sequences from the Internal Transcribed Spacers of nuclear ribosomal DNA (ITS1 and ITS2) to infer the phylogenetic relationships for representatives of the *Carduncellus-Carthamus* complex. Vilatersana et al. (2000) obtained strong bootstrap support for the removal of *C. caeruleus* from *Carthamus* and its placement in *Lamottea* in agreement with the classification of López-González (1989) (Table 1). Artificial crosses with *Lamottea caeruleus* (synonym of *Carthamus caeruleus*) with other species of *Carthamus* failed to produce seed except for a single cross with *C. leucocaulos* which produced a single sterile F_1 plant with low pollen viability (Ashri and Knowles, 1960; Estilai and Knowles, 1976; Estilai and Knowles, 1978) (Fig. 2-1). The poor fertility and difficulty of obtaining seeds from these crosses provides further evidence for the distant relationship of *L. caeruleus* to the other members of *Carthamus*. Phylogenetic studies using data from morphology (Lopez-

Gonzalez, 1989) and DNA sequences (Vilatersana et al., 2000) suggest that both *C. arborescens* and *C. rhiphaeus* should be removed from *Carthamus* and placed in the genus *Phonus* (Table 1). *Phonus arborescens,* crossed with *C. divaricatus*, *C. leucocaulos*, and several *Carthamus* species with n = 12 (Fig. 2-1) did not produce seed, further establishing the distance of *P. arborescens* from the rest of the genus (Ashri and Knowles, 1960; Estilai and Knowles, 1976, 1978). With the transfer of these perennial taxa, *C. arborescens* and *C. rhiphaeus,* from *Carthamus*, the remaining members of the genus are annuals (Vilatersana et al., 2000).

Vilatersana et al. (2000) removed the ITS sequences obtained from three polyploid species, *C. creticus* L., *C. lanatus* L., and *C. turkestanicus*, from their phylogenetic analysis because their inclusion decreased bootstrap support values. These authors interpreted this result as indicative of the hybrid origins of these species previously proposed by several authors (Ashri and Knowles, 1960; Schank and Knowles, 1964; Harvey and Knowles, 1965; Khidir and Knowles, 1970a, b; Estilai and Knowles, 1978). Thus, the phylogenetic relationships of these three polyploid taxa to the other species of *Carthamus* remain obscure.

Overall the phylogenetic inference of Vilatersana et al. (2000) based on DNA sequence data is congruent with the classification of López-González based on morphological data (1989) (Table 1). Further details about interspecific hybridization among the species of *Carthamus sensu* López-González (1989) are discussed and placed into an evolutionary context.

Interspecific hybridization within Carthamus

Hybridization with several wild species of *Carthamus* may have played a role in the evolution of *C. tinctorius* in the Mediterranean and Asia where they are sympatric (Ashri and Knowles, 1960; Schank and Knowles, 1964). Extensive empirical studies of interspecific hybridization of *C. tinctorius* with its wild relatives enable estimates of cross-compatibility, provide information to predict potential hybridization and have been used to infer phylogenetic relationships within *Carthamus* (Kadam and Patankar, 1942; Claassen, 1950; Deshpande, 1952; Ashri and Knowles, 1960; Ashri and Efron, 1964; Knowles and Schank, 1964; Schank and Knowles, 1964; Ashri and Rudich, 1965; Knowles, 1969, 1980; Imrie and Knowles, 1970; Khidir and Knowles, 1970a, b; Imrie and Knowles, 1971; Estilai and Knowles, 1976, 1978; Estilai, 1977; Heaton and Klisiewicz, 1981; Kumar, 1991; Jambhale, 1994).

Section Carthamus (Taxa with n = 12)

Several authors have considered *C. tinctorius*, *C. persicus* (syn. *C. flavescens*), *C. palaestinus* and *C. oxyacanthus* as separate species but they are more likely races of a biological species (Table 1; Ashri and Efron, 1964; Baker, 1970; Imrie and Knowles, 1970). The section *Carthamus sensu* López-González (1989) includes the four aforementioned species and *C. curdicus* and *C. gypsicola* (Table 1). They share a chromosome number of n = 12 (Deshpande, 1952; Ashri and Knowles, 1960; Knowles, 1989) and have been inferred as close relatives in phylogenetic studies utilizing data from morphology, cytogenetics and DNA sequences (Deshpande, 1952; Ashri and Rudich, 1965; Imrie and Knowles, 1970; Knowles, 1989; Vilatersana et al., 2000). Most species of section *Carthamus* (Table 1) have been artificially crossed to produce fertile F_1 and F_2

progeny (Fig. 2-1 and 2-2) (Deshpande, 1952; Ashri and Knowles, 1960; Ashri and Efron, 1964; Ashri and Rudich, 1965; Imrie and Knowles, 1970; Knowles, 1989). Natural (open pollinated) hybrids of *C. tinctorius* (BB) and *C. oxyacanthus* (BB) demonstrate hybrid vigor (Table 3) (Deshpande, 1952) and have been documented in both Pakistan and India where they are sympatric (Deshpande, 1952; Knowles, 1969; Knowles and Ashri, 1995) and also when they were grown together in a greenhouse (Knowles, 1969; Knowles and Ashri, 1995). Similar natural hybridization has been inferred between *C. tinctorius* (BB) and *C. palaestinus* (BB) in Israel where these species are sympatric based on morphological comparisons of material from this region (Ashri and Rudich, 1965; Knowles and Ashri, 1995). The recent phylogenetic study of Vilatersana et al. (2000) confirmed the close relationship of three of the species from section *Carthamus* (*C. tinctorius*, *C. oxyacanthus* and *C. gypsicola*). Hybridization experiments with *C. curdicus* and *C. gypsicola* of the section *Carthamus sensu* López-González (1989) have not been attempted (Knowles, 1989). However, the close phylogenetic relationship inferred by Vilatersana et al. (2000) for *C. gypsicola* with *C. tinctorius* and *C. oxyacanthus* might be indicative of the potential for cross-compatibility among these species.

Intersectional crosses

Carthamus lanatus (n = 22; $A_1A_1B_1B_1$) is thought to have originated as a result of a hybridization event between two species (allopolyploidy), one species having a chromosome number of n = 10 and the other n = 12, followed by a subsequent doubling of the chromosomes (Table 3) (Khidir and Knowles 1970b). *Carthamus lanatus* material from naturalized populations in California was examined by Ashri and Knowles (1960) and found to have regular pairing of chromosomes during meiosis. Hybrids between *C.*

lanatus and species with n = 10 (*C. glauca*, *C. leucocaulos*, and *C. dentatus*) and n = 12 (*C. oxyacanthus*, *C. palaestinus*, *C. persicus*, and *C. tinctorius*) had irregular pairing of chromosomes during meiosis I and produced infertile hybrids (Table 1; Figs. 2-1 and 2-2). A hybrid from a cross of *C. dentatus* with *C. lanatus* was obtained, but the fertility of this individual was not reported (Khidir and Knowles, 1970b). Attempts to observe homology of chromosomes during meiosis in hybrids with *C. lanatus* and other species of *Carthamus* have not revealed any potential parental species (Ashri and Knowles, 1960; Estilai and Knowles, 1976, 1978; Khidir and Knowles 1970b).

Crosses with *C. tinctorius* (n=12) and *C. lanatus* (n=22) produced sterile progeny (Fig. 2-1) (Ashri and Knowles, 1960; Heaton and Klisiewicz, 1981). These authors reported that hybrid embryos from crosses with these two species were viable but were unable to penetrate the pericarp during germination preventing them from growing into fertile plants. Heaton and Klisiewicz (1981) obtained hybrids from a cross of *C. tinctorius* and *C. lanatus* when either species was used as the female recipient. Further manipulations by Heaton and Klisiewicz (1981) were required to obtain fertile hybrid plants from these crosses. They treated the rescued embryos (n = 17) with colchicine causing a doubling of the chromosome number and producing an autopolyploid (n = 34). Ashri and Knowles (1960) obtained one infertile hybrid from a cross of *C. tinctorius* and *C. lanatus* and found incomplete pairing of chromosomes during meiosis I. The sterility associated with irregular meiosis may have prevented backcrossing of these hybrids with *C. tinctorius* (Ashri and Knowles, 1960; Heaton and Klisiewicz, 1981). Thus, the likelihood of a hybrid between cultivated safflower and *C. lanatus* surviving under natural conditions is highly unlikely.

Section Atractylis (polyploid taxa)

Khidir and Knowles (1970a) suggest that both *C. creticus* ($A_1A_1B_1B_1A_2A_2$) and *C. turkestanicus* ($A_1A_1B_1B_1A_3A_3$) are a product of independent historical hybridization events with an ancestral form of *C. lanatus* (n = 22; $A_1A_1B_1B_1$) and two different species with n = 10. Recent phylogenetic analysis of several molecular markers provides strong evidence for allopolyploid orgins of *C. turkestanicus* and *C. creticus* by ancient hybridization followed by chromosome doubling with *C. lanatus* and *C. glaucus* and *C. leucocaulos*, respectively (Vilatersana et al., 2007). Crosses of the hexaploids, *C. turkestanicus* (n = 32) and *C. creticus* (n = 32) yielded hybrids with several irregularities during meiosis including quadrivalents. The quadrivalent formation of chromosomes during meiosis was used to infer a reciprocal translocation and thus substantial genetic difference between these species (Khidir and Knowles, 1970a). The hybrids of these two hexaploid species produced both pollen and seed with low fertility (Fig. 2-1) (Ashri and Knowles, 1960; Khidir and Knowles, 1970a). These results substantiate a distant relationship between these two hexaploid species (Table 1), but does not rule out the possibility that an ancestor of *C. lanatus* (n = 22) and two different species (n = 10) hybridized to produce these distinct polyploid species.

Crosses of *C. lanatus* (n = 22) with either *C. creticus* or *C. turkestanicus* (n = 32) produced hybrids which formed 22 bivalent and 10 univalent chromosomes during meiosis I (Estilai and Knowles, 1978; Khidir and Knowles, 1970a, b). The analysis of alcohol dehydrogenase allozymes by Efron et al. (1973) demonstrated that *C. lanatus*, *C. creticus* and *C. turkestanicus* share a unique allele for one subunit of this enzyme not found in other species of *Carthamus*. The results of the hybridization and allozyme

studies further substantiate the idea that an ancestor of *C. lanatus* may have been one of the progenitors of the hexaploid (n = 32) members of *Carthamus*.

Khidir and Knowles (1970b) hypothesized that *C. creticus* is the product of a hybridization event between taxa similar to the modern *C. leucocaulos* and *C. lanatus*. Crosses with *C. leucocaulos* (n = 10) and *C. lanatus* (n = 22) produced hybrids (n = 16) that were highly similar morphologically to *C. creticus* (n = 32) especially when the chromosome number of the resulting hybrids was doubled artificially with colchicine. The F_1 plants from these crosses had close chromosome homology determined by pairing at meiosis I; however, authors reported that this could be due to autosynditic pairing of chromosomes from the polyploid *C. lanatus* (Ashri and Knowles, 1960; Khidir and Knowles, 1970a). These hybrids had poor pollen viability and did not produce viable seeds (Ashri and Knowles, 1960; Khidir and Knowles, 1970a). Geographic ranges of *C. leucocaulos*, *C. lanatus*, and *C. creticus* overlap in Crete, where they are difficult to distinguish morphologically (Table 2; Khidir and Knowles 1970a). In addition, Vilatersana et al. (2007) found strong support for this hypothesis using phylogenetic analysis of molecular markers. Grant noted (1971, pp. 52) that when hybrids and parental species exchange genes frequently, introgressive populations can be created and these populations may lack distinct morphological and ecological characteristics of the original species. The similarities among *C. creticus*, *C. lanatus* and *C. leucocaulos*, where sympatric, are indicative of current or historic gene flow or, alternatively, parallel evolution.

Evidence for the relationship between *C. lanatus* and *C. turkestanicus* comes from the results of hybridization experiments where all of the species with n = 10 were crossed

with *C. lanatus* (Fig. 2-1; Khidir and Knowles 1970b). These crosses produced F_1 hybrids with floral heads that resembled those of *C. turkestanicus* (n = 32) (Khidir and Knowles 1970b). The geographical ranges of *C. turkestanicus* and *C. lanatus* overlap in an area west of Turkey to Kashmir (Table 2). In this area, both species are difficult to distinguish from one another (Khidir and Knowles, 1970a), which may be indicative of an introgressive population between two close relatives in an area of sympatry (Grant, 1971).

Section Odonthagnathis (Taxa with n = 10 and 11)

A close relationship among the taxa with n = 10 was found from crosses between all of these species with one another (Fig. 2-1 and 2-2). The close relationship among the taxa with n = 10 are reflected in the classification of López-González (1989) (Table 1). Hybrids from these crosses were fertile, but crosses of these species with *C. tinctorius* produced sterile hybrids (Fig. 2-1) (Ashri and Knowles 1960; Schank and Knowles 1964). Hybrids from interspecific crosses of *C. divaricatus* (n = 11) and species with 20 somatic chromosomes resulted in good chromosome pairing during meiosis I, and partially fertile pollen and seed, which was interpreted as evidence for a close relationship between these taxa (Fig. 2-1) (Estilai and Knowles, 1978). Crosses with *C. tinctorius* and *C. divaricatus* produced self-incompatible hybrids that were fertile. The offspring from backcrossed of these hybrids with *C. tinctorius* had low fertility (Fig. 2-1) (Estilai and Knowles, 1976).

Estilai and Knowles (1978) placed the species with n = 10 into their section II (Table 2-1) and split this section into two groups based on reciprocal translocations that were observed during meiosis of interspecific crosses among these species. Specific status of

several varieties of *C. glaucus* has been proposed (Table 1). The subspecies of *C. glaucus sensu* Hanelt (1963) are all interfertile (Knowles and Schank, 1964); however, varieties from Palestine crossed with those from Iran and Syria yield hybrids with one or more translocations during meiosis. These results are indicative of regional variation within *C. glaucus*. The different regional variants of *C. glaucus* do not behave the same when used in interspecific crosses. For example, *C. glaucus* spp. *glaucus* from Syria and Iran crossed with *C. tenuis* produced fertile and infertile hybrids, respectively (Fig. 2-1). *Carthamus glaucus* spp. *alexandrinus* crossed with *C. tinctorius* produced infertile hybrids (Fig. 2-1) (Estilai, 1977), but other subspecies or varieties not yet tested may produce viable hybrids with cultivated safflower. *Carthamus glaucus* ssp. *glandulosus* is intermediate to and resembles artificial hybrids of *C. glaucus* ssp. *anatolicus* and *C. tenuis* (Ashri, 1973). Where *C. glaucus* spp. *glandulosus* is sympatric with either *C. glaucus* ssp. *anatolicus* or *C. tenuis* they are difficult to differentiate morphologically (Ashri, 1973). These observations and the results of artificial hybridization experiments led Ashri (1973) to conclude that the subspecies of both *C. glaucus* and *C. tenuis* were a single biological species in a hybrid swarm with some divergence over their geographic range. F_1 hybrids were obtained from *Carthamus glaucus* spp. *alexandrinus* crossed with *C. creticus* (not the reciprocal), and *C. glaucus* ssp. *anatolicus* crossed with *C. lanatus*, but their fertility was not reported (Khidir and Knowles, 1970b).

Uncertain placement (Carthamus nitidus)

Conflicting results have been reported for the relationship of *C. nitidus* with the other species of *Carthamus*. Crosses between *C. tinctorius* (n = 12) and *C. nitidus* (n = 12) produced sterile F_1 progeny (Fig. 2-1) (Knowles and Schank, 1964; Knowles, 1989).

Crosses of *C. nitidus* with both *C. dentatus* and *C. glaucus* (n = 10) failed to produce hybrids (Fig. 2-1) (Knowles and Schank, 1964). Based on these hybridization experiments, Knowles and Schank (1964) suggested that *C. nitidus* was more closely allied to section *Carthamus* (n = 12). However, the molecular study of Vilatersana et al. (2000) placed *C. nitidus* as the sister group of section *Odonthagnathis* (n = 10, 11; Table 2-1).

Based on the observed pairing behaviour of chromosomes during meiosis of interspecific hybrids, genomic formulas have been assigned to several members of *Carthamus* (Table 3) (Estilai and Knowles, 1978; Khidir and Knowles 1970b). These genomic formulas provide information about the interspecific compatibility among the genomes of *Carthamus* and may be used to develop hypotheses about the potential for movement of transgenes from interspecific hybrids leading to introgression.

Knowledge of hybridization potential among species of *Carthamus* (Fig. 2-1) and the species in the New World (Fig. 2-2) provide information about the biological potential of gene flow from a transgenic safflower variety to wild relatives. The compatibility of cultivated safflower with species from genera closely related to *Carthamus* (*Centaurea*, *Carlina*, *Atractylis*, *Phonus*, and *Carduncellus*) has not been studied extensively and only a few have been mentioned here. The possibility of hybridization with one of these taxa cannot be ruled out without empirical evidence. It should be noted that successful hybridization of two species experimentally (artificially) does not predict success in nature; however, it does establish potential cross-compatibility for hybridization and introgression (Ellstrand et al., 1999). Thus, I can hypothesize that *C. tinctorius* may hybridize with several wild relatives (Fig. 2-1 and 2-2) if both species were to flower in

synchrony and were growing close enough to one another for wind or insect vectors to transfer pollen between the plants.

GEOGRAPHIC DISTRIBUTION

Members of the genus *Carthamus* (Tour.) L. and *Carduncellus* Adans. are endemic to the eastern and western Mediterranean Basin, respectively (Table 2) (Knowles, 1969; Vilatersana et al., 2000).

Cultivated varieties of *C. tinctorius* have diverged because of cultivation by man in semi-isolated regions of the Mediterranean. Geographic areas with distinct centers of similarity for cultivated safflower, and the generalized characteristics of these varieties have been described (Ashri et al., 1975; Knowles, 1969). Both Knowles (1969) and Ashri et al. (1975) noted that these groupings were based on generalizations and that gene flow by introductions of cultivars from different regions was ongoing and prevents complete divergence. Knowles (1969) and Ashri et al. (1975) both noted that cultivated safflower in India was morphologically homogeneous and suggested that the Indian populations have been isolated from the others for several generations (Knowles and Ashri, 1995).

Cultivation of safflower in the New World began in 1899, but it was not commercially grown until the early 1950s (Knowles, 1958; 1989). In the USA, *C. tinctorius* has been reported to have escaped cultivation in Arizona (Keil, 2006), California (Hickman, 1993; Keil, 2006; Munz, 1968), Colorado (Keil, 2006), Idaho (Keil, 2006), Illinois (Keil, 2006), Iowa (Keil, 2006; Rydberg, 1971), Illinois (Henry, 1992; Keil, 2006), Kansas (Gates, 1940; Keil, 2006; Rydberg, 1971), Massachusetts (Keil, 2006), Montana (Keil, 2006), Nebraska (Keil, 2006), New Mexico (Keil, 2006; Martin and Hutchins, 1981), North Dakota (Keil, 2006), Ohio (Keil, 2006; Vincent and Cusick,

1998) Oregon (Keil, 2006), Utah (Keil, 2006; Shaw, 1989) Washington (Keil, 2006) (Table 2). In Canada it has been reported in Alberta and British Columbia (Keil, 2006).

Four wild safflower relatives have been introduced in some areas of the New World (Table 2) (Hickman 1993). *Carthamus oxyacanthus* (n = 12) and *C. lanatus* have been documented as being naturalized in Oregon and Florida (Hickman 1993) and is known from a single collection in California in 1978 (Keil, 2006). *Carthamus lanatus* and *C. leucocaulos* (n = 20) have both been documented in Texas and have been collected from Argentina and Chile (Correll and Johnston, 1970; Hickman, 1993; Keil, 2006; Marticorena and Quezada, 1985). An infestation of C. leucocaulos in one county in California was reported and eradicated in 1990 (Keil, 2006). *Carthamus creticus* (n = 32) and *C. lanatus* (n = 22) have been reported in California (Hickman, 1993; Hoover, 1970; Munz, 1968; Munz and Keck, 1968). *Carthamus lanatus* has been a weed in California since 1891 (Fuller, 1979). Kessler (1987) documented *C. lanatus* as a serious weed growing in an isolated area in Oklahoma. *Carthamus lanatus* has also been collected in Arizona, Massachusetts, New Jersey and Oregon (Keil, 2006).

BIOLOGY AND ECOLOGY

Information is limited on the biology and ecology of most of the species of *Carthamus*, however, some species have been studied as crops or weeds.

Carthamus tinctorius

Carthamus tinctorius is predominantly a self-compatible weedy annual thistle, with branched upright stems of 30 to 150 cm in height with terminal flowers, a deep taproot with laterals, and an inflorescence with a dense capitulum with green ovoid involucral

bracts (Smith, 1996). Pollination of *C. tinctorius* in Nebraska was facilitated by various insect vectors including *Halictus pictus* (sweat or mining bees), *Agapostemon radiatus* (sweat or mining bees) and *Chauliognathus basalis* (soldier beetles) and in California primarily by *Apis mellifera* (honeybees; Claassen 1950). Smith (1996) stated that *Carthamus tinctorius* does not frequently establish itself outside of human cultivated areas; however, feral populations have been documented in several US states and two Canadian provinces (Gates, 1940; Henry, 1992; Hickman, 1993; Keil, 2006; Martin and Hutchins, 1981; Rydberg, 1971; Shaw, 1989). The longevity of these escapes has not been documented. Knowles (1989) outlined three types of environments suited to growing non-cultivated *C. tinctorius*, all similar to its native Mediterranean region. All three environments were characterized by heavy soils with rains before or just after the seeds are sown followed by a dry period during the later stages of growth.

Carthamus lanatus

Carthamus lanatus (n = 22) is a self-compatible, shallow rooted, annual or facultative biennial (Groves and Kaye, 1989), with upright stems from 0.1 m to 2 m in height. It has a rosette of leaves reaching 15 cm in diameter (Kessler, 1987; Peirce, 1992). Seeds are not developed for wind dispersal; however, the pappus facilitates flotation in water and adherence to animal fur and to clothing (Peirce, 1992). Pollination of this species is facilitated by honey bees (*Apis mellifera*) (Peirce, 1992). In Australia, seeds of *C. lanatus* are dispersed by hay, chaff, grain seed contamination and by attachment to sheep wool (Kessler, 1987). Australian *C. lanatus* produces 70 to 177 viable seeds per plant (Peirce, 1992). Seeds can remain viable for eight to ten years (Quinlivan and Pierce, 1969), but most germinate within three years (Peirce, 1992). In Oklahoma, *C. lanatus* flowers during

the late summer or early fall when *C. tinctorius* is being harvested (Kessler, 1987). In California it flowers between July and August (Kessler, 1987). *Carthamus lanatus* establishes in areas where the soil has been disturbed or perennial grasses have been thinned or removed. This species populates areas that have experienced drought or over-grazing (Peirce, 1992). It is weedy in pastures and cereal crops in Australia (Peirce, 1990).

Carthamus lanatus has been introduced to South Africa, North America and Australia (Peirce, 1992). In Australia it has had control legislation since 1887 (Kessler, 1987). In New South Wales, this species has been considered one of the most serious, difficult and costly thistle weeds (Briese, 1988), causing yield losses of up to 50 to 70% of cereal crops (Peirce, 1992). In Victoria, however, it is not recognized as an aggressive weed (pers. com. Brooke Thompson). The distribution of this species in Australia is predominantly near cereal-growing districts, especially in southeastern regions of the continent (Kessler, 1987). In the US, *C. lanatus* has been identified as a potentially serious weed in Oklahoma, where a single, isolated, population has been difficult to eradicate (Kessler 1987). Cattle and sheep avoid grazing on *C. lanatus* (Kessler, 1987; Peirce, 1992).

SEED BIOLOGY OF SOME *CARTHAMUS* SPECIES

Successful introduction and naturalization of a *Carthamus* species in the New World depends on their ability to reach maturity reproduce within the growing season and whether the seeds can survive adverse environmental conditions.

Some research has been published on the seed biology of some species of *Carthamus*, which may provide insights into the longevity and survivorship in the environment. Kessler (1987) determined that germination of *C. lanatus* seeds were reduced or prevented by moisture stress (limited at –0.25 MPa and no germination at –0.5 MPa) and were long-lived in the soil. The optimal germination conditions for seeds of *C. lanatus* were found to be 8 hours at 20 C° and 16 hours at 10 C° (Groves and Kaye, 1989). Seeds of *C. lanatus* were unaffected by freezing at -16 C° while other thistle species (not members of *Carthamus*) were injured. Experimental varieties of *C. tinctorius* have been able to withstand temperatures as low as -15 C° following a hardening period (Knowles and Ashri, 1995; Zimmerman and Buck, 1977). Seeds of *C. persicus* were able to withstand temperatures of -16 C° for four hours with about 50% of the seeds remaining viable. Crosses with *C. tinctorius* and *C. persicus* produced hybrid plants with cold tolerance similar to the latter species (Zimmerman and Buck, 1977). Gupta and Murty (1986) reported seeds of *C. oxyacanthus* to be resistant to low and high humidity and both high and fluctuating temperatures. The ability of seeds from *C. tinctorius* and its wild relatives to withstand extreme environmental conditions is not well understood and requires further research.

OUTCROSSING RATES

Cross-pollination of safflower plants is facilitated predominantly by insects, and wind can move pollen short distances (up to ca. 122 cm) between plants grown close together (Ashri and Rudich, 1965; Claassen, 1950). Bees and other flying insects may contribute to gene flow among *C. tinctorius* and its wild relatives over relatively large distances (Claassen, 1950; Kadam and Patankar, 1942). Claassen (1950) grew (safflower) plants in

close proximity (less than 122 cm) and found levels of outcrossing that varied from 5 to 45% in some cultivars, but most cultivars were found to have less than 10% outcrossing (Claassen, 1950; Kadam and Patankar, 1942; Knowles, 1969). The amount of outcrossing was found to be highly variable among safflower cultivars and was partially controlled by heredity (Claassen, 1950). Among individual plants from various safflower lines the range in cross-pollination was 0 to 100% (Claassen, 1950). The variation in outcrossing rates among different cultivars of *C. tinctorius* (Claassen, 1950) means that empirical measurement of outcrossing frequency should be conducted under different environmental conditions for cultivars of interest. The variation in outcrossing rate between cultivars means that this value should be experimentally determined for cultivars being considered for the production of plant-made pharmaceuticals.

ECOLOGICAL NICHE

The niche occupied by a wild crop relative is often indicative of the spatial relationship between a wild crop relative and crop and thus, the potential for introgression. *Carthamus tinctorius* is the product of selection by humans in an agricultural environment, whereas *C. palaestinus*, *C. persicus* (syn. *C. flavescens*) and *C. oxyacanthus* are considered weeds in areas disrupted by human and agricultural activities (Baker, 1965; Imrie and Knowles, 1970). *Carthamus tinctorius* has a shorter duration of the rosette stage selected to enhance earlier maturity of the crop. In addition, the heads of *C. tinctorius* are shatter resistant and generally lack a pappus, ensuring that seeds are easily harvested (Ashri and Efron, 1964). *Carthamus persicus* is a weed of wheat fields, pastures and roadsides of cooler climates in eastern Iraq, Lebanon, Syria and Turkey (Imrie and Knowles, 1970; Knowles and Ashri, 1995; Smith, 1996). Its weediness is attributed to a delayed rosette

stage with aerial stem and flower development after the harvest of cereal crops, thus ensuring its seeds are distributed back to the field. The seeds of *C. persicus* are easily released by shattering of the heads and have a pappus, both traits advantageous for wind dispersal. The most broadly distributed weedy species of *Carthamus* in the Mediterranean is *Carthamus oxyacanthus*. It has seeds that are released by shattering of the heads, but the seeds have a reduced pappus (Ashri and Efron, 1964). This species is found in subtropical regions of western Iraq, Iran, north-west India, through-out Kazakhastan, Turkmenistan, and Uzbekistan (Table 2) (Knowles and Ashri, 1995; Smith, 1996). *Carthamus palaestinus* has seeds that are released by shattering of the heads (Ashri and Efron, 1964) and is only known from desert regions of western Iraq, Israel, and Jordan (Knowles and Ashri, 1995; Smith, 1996).

CONCLUSION

A transgenic safflower variety (*Carthamus tinctorius* L.) has been derived by the fusion of a gene encoding a protein based pharmaceutical with the protein oleosin. The purification of a protein from the mature oil rich seeds (*ca.* 30 - 40% of the seed dry weight) of safflower has the potential for economic success (Smith, 1996) and the isolation of oleosin from safflower seed has been documented (Lacey et al., 1998). A concern with this new technology is the escape of transgenes into the environment. Gene flow from the crop to one of its wild relatives could lead to introgression, the stable incorporation of a transgene. Direct environmental consequences include the loss of herbicidal control of weed relatives. More importantly, there is a public perception that transgenes pose unknown risks and therefore must be contained.

Most domesticated plants have been reproductively isolated from their natural populations for less than *ca.* 1000 generations (Ellstrand et al., 1999). In this short time, it is unlikely that complete reproductive isolation has occurred. Gene flow at low rates among populations can reduce differentiation generated by genetic drift, mutations, and natural selection, which in turn prevents divergence of populations from one another (Arnold, 1997; Ellstrand et al., 1999). Although *C. tinctorius* has been cultivated for more than 1000 years (Knowles and Ashri, 1995; Smith, 1996), interspecific hybridization experiments have shown that *C. tinctorius* can be crossed with several wild relatives to produce fertile progeny (Figs. 2-1 and 2-2) (Ashri and Knowles, 1960; Estilai and Knowles, 1978; Heaton and Klisiewicz, 1981; Imrie and Knowles 1970, 1971; Khidir and Knowles 1970a, b; Schank and Knowles, 1964; reviewed by Kumar 1991).

Potential recipients of nuclear encoded transgenes from a cultivated plant-made pharmaceutical with safflower in the New World include *C. tinctorius* escapes from cultivation and four naturalized wild relatives (*C. creticus*, *C. lanatus*, *C. leucocaulos* and *C. oxyacanthus*). Of these wild species, only *C. oxyacanthus* and *C. creticus* have been shown to produce viable hybrid offspring when crossed with cultivated *C. tinctorius* and then only under some conditions (Figs. 2-1 and 2-2). Hybrids of *C. tinctorius* and *C. lanatus* were not viable without the use of embryo rescue and chromosome doubling techniques (Fig. 2-1) (Ashri and Knowles, 1960; Heaton and Klisiewicz, 1981). Thus, this hybrid is not likely to survive outside of a laboratory setting. *Carthamus tinctorius* has been naturalized in parts of the US, thus making it another potential transgene recipient and a possible intermediary for transgene movement to wild species. However, the frequency of gene exchange would depend on the outcrossing frequency of both

naturalized safflower cultivars and the transgenic crop. Gene flow has the potential to occur between a transgenic crop of safflower (*C. tinctorius*) and either *C. oxyacanthus* or *C. creticus* in several regions of the New World where these species are sympatric, including most regions of the New World currently utilized to cultivate safflower (Fig. 2-2; Tab. 2-2).

Mitigation of the potential for transgene escape can best be achieved by avoiding the cultivation of a transgenic *C. tinctorius* in areas where feral safflowers occur and where cross-compatible weedy relatives are suspected. The lack of wild and weedy relatives of cultivated *C. tinctorius* in most of the western prairie regions of Canada may provide a safe region to grow a plant-made pharmaceutical safflower than more southern locations in the New World. Several regions in the southern US could also provide isolated locations for the cultivation of transgenic safflower. Cultivation of this new crop is not suitable in Asia and the middle-east where several wild and weedy relatives are thought to currently hybridize frequently with cultivated safflower (Ashri and Rudich, 1965; Deshpande, 1952; Knowles and Ashri, 1995).

Table 2-1. Comparison of the *Carthamus* intrageneric classification schemes by Hanelt (1963), Estilai & Knowles (1976) and López-González (1989).

Hanelt (1963)	Estilai & Knowles (1976)	López-González (1989)
Section *Carthamus* (n = 12)	**Section I** (n = 12)	**Section** *Carthamus* (n = 12)
C. curdicus Hanelt	*C. oxyacanthus* Bieb.	*C. curdicus* Hanelt
C. gypsicola Ilj.	*C. palaestinus* Eig.	*C. gypsicola* Ilj.
C. oxyacanthus Bieb.	*C. persicus* Willd.[1]	*C. oxyacanthus* Bieb.
C. palaestinus Eig.	*C. tinctorius* L.	*C. palaestinus* Eig.
C. persicus Willd.[1]	**Section II** (n = 10)	*C. persicus* Willd.[1]
C. tinctorius L.	*C. alexandrinus* Ascher [2]	*C. tinctorius* L.
Section *Odonthagnathis (n = 10)*	*C. dentatus* Vahl.	**Section** *Odonthagnathis (n = 10, 11)*
C. dentatus (Forssk.) Vahl.	*C. leucocaulos* Sibth. *et* Sm.	*C. boissieri* Halácsy
spp. *dentatus* Hanelt	**Section III** (n = 22)	*C. dentatus* (Forssk.) Vahl.
spp. *ruber* (Link) Hanelt	*C. lanatus* L.	*C. divaricatus* Beguinot & Vacc.[3]
Section *Atractylis* (n = 22, 32)	**Section IV** (n = 11, 32)	*C. glaucus* Bieb.[2]
C. lanatus L.	*C. creticus* L.[4]	*C. leucocaulos* Sibth. *et* Sm.
spp. *lanatus* Hanelt	*C. divaricatus* Beguinot & Vacc.[3]	*C. tenuis* (Boiss. & Bl.) Bornm.
spp. *creticus* (L.) Holmb.[3,4]	*C. turkestanicus* Popov	**Section** *Atractylis* (n = 22, 32)

spp. *montanus*

spp. *turkestanicus* Hanelt

Section *Thamnacanthus*

C. arborescens L.[5]

C. rhiphaeus F. -Q. et Pau[8]

C. X *martis* F. -Q.[8]

Section *Lepidopappus* Hanelt

Series *Lepidopappi* (n = 10)

C. glaucus Beib.

spp. *glaucus*

spp. *glandulosus* Hanelt

spp. *anatolicus* Hanelt

spp. *alexandrinus* Hanelt[2]

C. boissieri Halácsy

C. tenuis (Boiss. & Bl.) Bornm.

spp. *tenuis*

spp. *gracillimus* (Rech. f.) Hanelt

spp. *foliosus* (Boiss.) Hanelt

Other (n = 12)

C. arborescens L.[5]

C. nitidus Boiss.[6]

Taxa removed from *Carthamus*

Carduncellus *caeruleus* L. (n = 12)[7]

C. creticus L.[4]

C. lanatus L.

C. turkestanicus Popov

Uncertain placement (n = 12)

C. nitidus Boiss.[6]

Femeniasia balearica Susanna

Taxa removed from *Carthamus*

Lamottea caeruleus (L.) Pomel[7]

Phonus rhiphaeus G. López[8]

Phonus arborescens (L.) G. López[5]

Series *Leucauli* (n = 10, 12)

C. leucocaulos Sibth. *et* Sm.

C. X (?) *rechingeri* Davis

C. nitidus Boiss.[6]

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[1]*C. flavescens* is a basionym for the currently recognized *C. persicus.*
[2]Hanelt (1963) and López-González (1989) considered *Carthamus alexandrinus* as a member of *C. glaucus.*
[3]Hanelt recognized *C. lanatus* ssp. *creticus* var. *divaricatus*, whereas Estilai & Knowles (1976) and López-González (1989) recognized *C. divaricatus.*
[4]*Carthamus creticus* is a synonym of *C. baeticus* & of *C. lanatus* ssp. *creticus.*
[5]*Carthamus arborescens* was included in *Phonus* by López-González (1989), and the molecular study of Vilatersana *et al.* (2000) provided strong evidence for the exclusion of this species from *Carthamus* and *Carduncellus.*
[6]*Carthamus nitidus* was considered by López-González (1989) to constitute a possible monospecific section. Vilatersana et al. (2000) found *C. nitidus* as the sister of several species of *Odonthagnathis* justifying the placement of *C. nitidus* in either this section or in its own section.
[7]López-González (1989) moved *Carthamus caeruleus* to the genus *Lamottea.*
[8]*Carthamus rhiphaeus* Font Quer & Pau was changed to *Phonus rhiphaeus.* The hybrid *Carthamus* X *martis* Font Quer in Hanelt (1963) was considered a synonym for *Phonus arborescens* X *Phonus rhiphaeus* by López-González (1989).

Table 2-2. Geographical distributions of *Carthamus sensu* López-González (1989).

Taxon	Known location (endemic or naturalized)
Carthamus L.	
C. curdicus Hanelt	N. Iran only
C. gypsicola Ilj.	N. W. Iran, N. Iraq, S. W. Kazakhastan, Leb., Turkmenistan, Uzbekistan, Syria, and Tur. (Caspian to the Aral Sea)
C. oxyacanthus Bieb.	Aus., Caucasus, S. E. Eur., N. W., India to central Iraq (including Kash.), Iran, Israel, Jordan, S. W. Kazakhastan, Pak., USA (CA, FL & OR), Syria, Turkmenistan, S. Tur., and Uzbekistan
C. palaestinus Eig	S. Israel
C. persicus Willd.	W. Iran, Israel, N. Iraq, Leb., Syria, and Tur
C. tinctorius L.	Widely cultivated; probable origins in W. Asia [a]
***Odonthagnathis* (DC.) Hanelt**	
C. boissieri Halácsy	Unknown
C. dentatus (Forssk.) Vahl	Balkans and Aegean region (including Crete, Cyprus and Gr.), Aus., and Tur. to Iran
C. divaricatus Beguinot & Vacc.	Only known from coastal Libya
C. glaucus Bieb.	N. W. Afr. to Egypt along the Mediterranean coast to Israel, Azerbaijan, Tur. to Kash., W., Crete, E. Eur., Iran, Jordan, Leb., Libya, Syria, and S. Ukraine
C. leucocaulos Sm.	Aus., Islands of the Aegean Sea and adjacent Gr. (incl. Crete), S. E. Eur., S. Afr., Aus., Arg., and USA (CA and OR)

C. tenuis (Boiss. & Bl.) Bornm. Cyprus, Egypt, Israel, Leb. and S. W. Tur.

Uncertain placement

C. nitidus Boiss. Israel, Jordan, Leb., Syria, and Palestine

Atractylis Reichenb.

C. creticus L. Aus., Canada (BC), Crete, Egypt, Iraq, USA (CA, NE, OR & SC), Rhodes, and Tur.

C. lanatus L. Tur. to Kash. (Afg.), Canary Islands, S. Eur., N. and S. Afr., Aus., Arg., Chile and USA (AZ, CA, FL, NJ, OK, OR and TX)

C. turkestanicus Popov W. Tur. to Kash., Iraq, Pak., Afg., and Eth.

[a]An extensive evaluation of germplasm from all of the locations of the world that *C. tinctorius* was known to be cultivated was conducted by Ashri et al. (1975).
Symbols: Afg. Afganistan, Afr. Africa, Aus. Australia, Arg. Argentina, Eth. Ethiopia, Eur. Europe, Gr. Greece, Kash. Kashmir, Leb. Lebanon, Pak. Pakistan, Tur. Turkey, USA United States of America, AZ Arizona, CA California, FL Florida, MA Massachusetts, NJ New Jersey, OK Oklahoma, OR Oregon, TX Texas, W. west, E. east, N. north, and S. south.

Table 2-3. Self-compatibility and genomic formula for the species of *Carthamus* sensu López-González (1989).

Taxon	Self-compatibility [a]	Genomic formula [b]
Section *Carthamus* (2n = 24)		
C. curdicus Hanelt	compatible	—
C. gypsicola Ilj.	compatible	—
C. oxyacanthus Bieb.	both known	BB
C. palaestinus Eig.	compatible	B_1B_1
C. persicus Willd.	incompatible	B_1B_1
(syn. *C. flavescens* Spreng.)		
C. tinctorius L.	compatible	BB
Uncertain placement (2n = 24)		
C. nitidus Boiss.	compatible	—
Section ***Odonthagnathis*** (DC.)		
Hanelt (2n = 20, 22)		
C. boissieri Halácsy	unknown	—
C. dentatus (Forssk.) Vahl.	incompatible	A_1A_1
C. divaricatus Beguinot & Vacc.	incompatible	—
C. glaucus Bieb.	unknown	AAA_3A_3

C. leucocaulos Sm.	compatible	A_2A_2 [c]
C. tenuis (Boiss. & Bl.) Bornm.	unknown	—
Section ***Atractylis*** Reichenb. (2n = 44, 64)		
C. creticus L.	compatible	$A_1A_1B_1B_1A_2A_2$
C. lanatus L.	compatible	$A_1A_1B_1B_1$
C. turkestanicus Popov	compatible	$A_1A_1B_1B_1A_3A_3$
Previously considered members of *Carthamus* / *Carduncellus* (2n = 24)		
Lamottea caeruleus (L.) Pomel	unknown	unknown
Phonus arborescens (L.) G. López	unknown	unknown
Phonus rhiphaeus G. López	unknown	unknown

[a] Where tested the incompatibility system has been shown to be of a sporophytic type (Knowles 1989; Imrie and Knowles 1971).
[b] Genomic formulas are from Khidir and Knowles 1970b (also see review by Kumar 1991)
[c] The genomic formula for *C. leucocaulos* was changed to AA by Estilai and Knowles (1978).

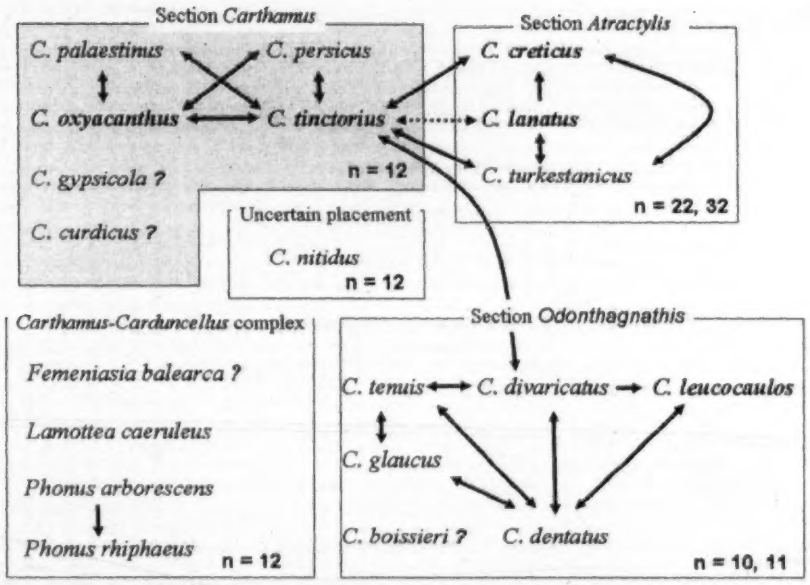

Figure 2-1. Summary of the artificial interspecific crosses that resulted in fertile progeny (see text for references). Solid lines indicate fertile F_1 hybrids with viable seed production. Dotted lines indicate hybridization occurred, but the F_1 hybrids could not be obtained without embryo rescue and / or treatments with colchicine. Arrows indicate the direction of the cross (male → female). The shaded box contains taxa with n = 12, which are all members of section *Carthamus* except the unplaced *C. nitidus*. Species in bold are naturalized to locations in the New World. [a]*Carthamus* glaucus contains several subspecies (Table 1), which are all interfertile (Knowles and Schank 1964), however, interspecific crosses with *C. glaucus* spp. *glaucus* from different geographic regions do not produce the same results. [b]Crosses with *C. divaricatus* and *C. tinctorius* produced self-incompatible hybrids, which had low fertility when backcrossed with *C. tinctorius*. [c]Crosses of *C. gypsicola*, *C. boissieri*, *C. curdicus* and members of the newly discovered *Femeniasia* to other species of *Carthamus* have not been published to date.

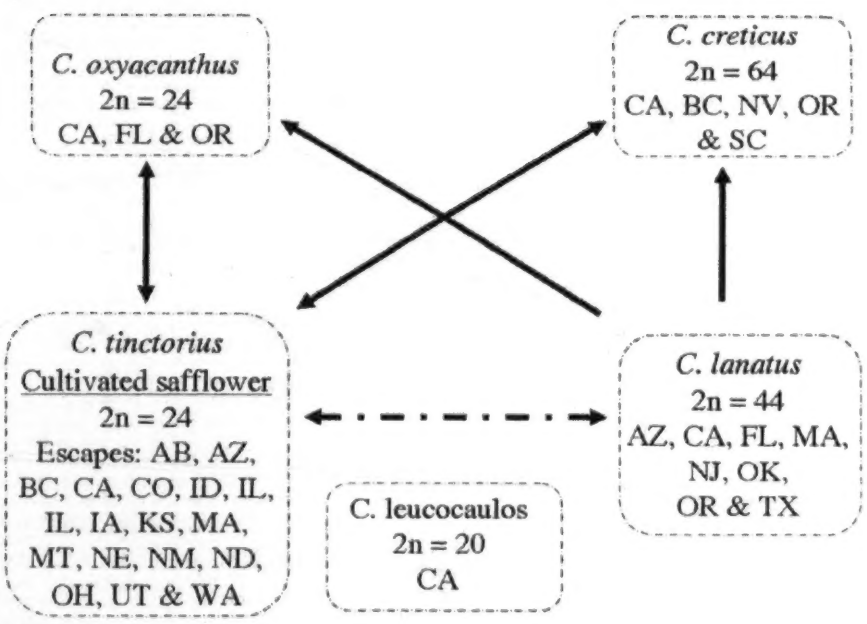

Figure 2-2. Summary of interspecific hybridization experiments for taxa found in the Canada and the United States (see text for references). Solid lines indicate fertile F_1 hybrids with viable seed production. Dotted lines indicate hybridization occurred, but F_1 hybrids could not be obtained without embryo rescue and / or treatments with colchicine. Arrows indicate the direction of the cross (male → female). Symbols: AB Alberta, AZ Arizona, BC British Columbia, CA California, CO Colorado, FL Florida, ID Idaho, IL Illinois, IA Iowa, KS Kansas, MA Massachusetts, MT Montana, NE Nebraska, NV Nevada, NM New Mexico, ND North Dakota, OH Ohio, OK Oklahoma, OR Oregon, SC South Carolina, TX Texas, UT Utah and WA Washington.

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Chapter 3: Issues of ferality and domestication for safflower intended for plant made pharmaceuticals

A section of the book chapter: André Bervillé, Catherine Breton, Ken Cunliffe, Henri Darmency, Allen G. Good, Jonathan Gressel, Linda M. Hall, Marc A. McPherson, Frédéric Médail, Christian Pinatel, Duncan A. Vaughan, and Suzanne I. Warwick (2004) Issues of ferality or potential for ferality in oats, olives, the pigeon-pea group, ryegrass species, safflower, and sugarcane. *In* Crop Ferality and volunteerism: a threat to food security in the transgenic era?

SAFFLOWER: FERALITY IN A PLANT MADE PHARMACEUTICAL PLATFORM

Cultivated safflower (*Carthamus tinctorius* L.) is being evaluated as a biological platform for the field production of high value plant-made pharmaceuticals (PMPs). The need to differentiate crops containing PMPs from food and feed crops has increased the interest of the pharmaceutical/biotechnology sector in using minor crops as a platform in Canada, including tobacco (*Nicotiana tabacum* L.), alfalfa (*Medicago* sp. L.), white clover (*Trifolium angustifolium* L.), Ethiopian mustard (*Brassica carinata* A. Braun), white mustard (*Sinapis alba* L.), flax (*Linum usitatissimum* L.), and safflower (*Carthamus tinctorius* L.) (Anonymous, 2007).

Biosafety concerns for PMPs are similar to traditional transgenic crops, including outcrossing to other non-transgenic varieties or wild and weedy relatives, the need to maintain isolation distances from cross-compatible species, segregation of PMP from

food or feeds and the feral nature of the crop. Because PMP products may have biological activity, unlike crops with input traits such as herbicide resistance, the impact of inadvertent environmental release and contamination of the food or feed system may be greater (Ma et al., 2003).

Safflower has been grown extensively in the Mediterranean and is thought to have originated in the Euphrates Basin (Knowles, 1989; Smith, 1996). Hybridization of safflower with sympatric (spatially) wild relatives may have played a significant role in the evolution of *Carthamus* L. and cultivated safflower in the Mediterranean (Ashri and Knowles, 1960; Schank and Knowles, 1964). Hybridization between and weedy relatives could facilitate a transfer of PMP or feral genes. The resulting hybrids could facilitate the introgression of a transgene into wild or weedy populations or the formation of feral populations. Safflower, originally grown for dye production (carthamine), is currently grown for the extraction of oil for human consumption and for birdseed.Safflower was first cultivated in the New World in 1899 and was commercially grown in the U.S. during the early 1950s (Knowles, 1958; Knowles, 1989). In recent years, approximately 80,000 ha per annum of was been harvested in the U.S. In Canada, less than 1000 ha have been harvested annually (Anonymous, 2002; Anonymous, 2007). Feral populations, volunteer that have become established in the agroecosystem, have been reported in the U.S. (Gates, 1940; Henry, 1992; Hickman, 1993; Martin and Hutchins, 1981; Rydberg, 1971; Shaw, 1989), and Canada (Keil, 2006). Thus, this crop may have the potential to de-domesticate under certain production conditions. In this review, I will discuss the implications of growing transgenic containing a plant-made pharmaceutical and the potential for feral populations to increase in both the U.S. and Canada.

PLATFORM FOR PLANT MADE PHARMACEUTICALS

'Centennial', a cultivated variety of safflower has been genetically engineered to express two nuclear encoded gene cassettes. The first is a selective marker, for glufosinate-ammonium resistance, and the second is a trait generated by the fusion of a gene encoding a PMP with the oil seed transmembrane protein oleosin (Lacey et al., 1998). The oleosin fusion protein enables the production and subsequent isolation of the PMP from oilseed crops (Moloney, 2000; Parmenter et al., 1995). This has previously been demonstrated in safflower (Lacey et al., 1998), where the isolation of the PMP from mature, oil rich safflower seeds (approximately 30 to 40% of the seed by dry weight) (Smith, 1996) has economic potential.

The selective marker, glufosinate resistance, is unlikely to confer a selective advantage to volunteers or feral populations because it is used only on a few crops and is not used in ruderal areas such as roadsides and waste areas. The fitness consequences of the PMP genes have not yet been determined experimentally.

Safflower, systematics, biology, biogeography

Systematics and hybridization potential between cultivated safflower and its wild relatives

Cultivated safflower is a member of the Asteraceae (Compositae), tribe Cardueae (thistles), and subtribe Centaureinae (Kumar, 1991; Vilatersana et al., 2000). The genus *Carthamus* L. has 16 recognized species *sensu* (Lopez-Gonzalez, 1989). It is a member of the *Carthamus-Carduncellus* complex that consists of several closely related genera including *Carduncellus* Adans., *Femeniasia* Susanna, *Lamottea* Pomel, and *Phonus* Hill

(Vilatersana et al., 2000). The cross-compatibilities among most of the members of *Carthamus* and some closely related species from the *Carthamus-Carduncellus* complex have been evaluated (Ashri and Knowles, 1960; Ashri and Efron, 1964; Ashri and Rudich, 1965; Claassen, 1950; Deshpande, 1952; Estilai and Knowles, 1976; Estilai, 1977; Estilai and Knowles, 1978; Heaton and Klisiewicz, 1981; Imrie and Knowles, 1971; Imrie and Knowles, 1970; Jambhale, 1994; Kadam and Patankar, 1942; Khidir and Knowles, 1970a; Khidir and Knowles, 1970b; Knowles and Schank, 1964; Knowles, 1969; Knowles, 1980; Kumar, 1991; Schank and Knowles, 1964). These papers have been reviewed (McPherson et al., 2004) and their findings summarized in Figure 3-1. Cultivated safflower and the other members of the section *Carthamus* have a chromosome number of n = 12 (Fig. 3-1). The ease of crossing and obtaining viable offspring among the members of this section of the genus has led several authors to consider these species (cultivated safflower, *Carthamus persicus* Willd., *Carthamus palaestinus* Eig, and *Carthamus oxyacanthus* Bieb.) to be a biological race rather than separate species (Ashri and Efron, 1964; Imrie and Knowles, 1970). Genomic formulas for some species in the genus *Carthamus* have been determined (Estilai and Knowles, 1978; Lacey et al., 1998). These formulas could be used to infer the potential for introgression of a nuclear encoded transgene from PMP safflower to a wild relative.

Vigorous hybrids from natural crosses of safflower (genomic formula BB) and *C. oxyacanthus* (BB) have been documented in both glasshouse studies when grown together and in fields in Pakistan and India, where the two species were sympatric (Deshpande, 1952; Knowles, 1969; Knowles and Ashri, 1995) (Fig. 3-1). Hybrids of safflower (BB) and *C. palaestinus* (B_1B_1) have been found in Israel where these species

were sympatric (Knowles, 1969; Knowles and Ashri, 1995) (Fig. 3-1). Artificial crosses of safflower with *C. persicus* (B_1B_1) have produced fertile F_1 and F_2 progeny (Imrie and Knowles, 1970) (Fig. 3-1). Hybridization experiments with *Carthamus gypsicola* Ilj., *Carthamus curdicus* Hanelt, and the newly discovered *Femeniasia balearica* Susanna have not yet been conducted. Numerous attempts to cross *C. nitidus* Boiss. (n = 12) with other members of the section *Carthamus* have not produced fertile hybrids (Knowles and Schank, 1964; Knowles, 1989) (Fig. 3-1). Safflower has also been crossed with four species outside of the section *Carthamus* to produce viable hybrids. A single cross of *C. divaricatus* Beguinot and Vacc. (n = 11; Section Odonthagnathis) with safflower was obtained but the offspring from backcrosses with these hybrids and safflower had low fertility (Estilai and Knowles, 1976) (Fig. 3-1). Crosses between safflower and *C. lanatus* L. ($A_1A_1B_1B_1$; n = 22; Section Atractylis) did not produce viable offspring without treatments with colchicine and embryo rescue (Fig. 3-1). Thus, the probability of a hybridization event between these two species producing viable offspring in nature is relatively low. Crosses with safflower and two members of Section Atractylis, *C. creticus* L. ($A_1A_1B_1B_1A_2A_2$; n = 32) and *C. turkestanicus* Popov ($A_1A_1B_1B_1A_3A_3$; n = 32), produced viable offspring (Fig. 3-1). The three recognized members of Atractylis are cross-compatible (Fig. 3-1). Thus, a transgene could introgress from PMP safflower to a weedy relative and spread to other species via hybridization.

***Carthamus*: Biology and Biogeography**

Carthamus oxyacanthus and *C. persicus* are weeds of wheat fields and roadsides of cooler climates (Ashri and Efron, 1964; Imrie and Knowles, 1970; Knowles and Ashri, 1995; Smith, 1996). *Carthamus palaestinus* is a wild relative found in desert regions of

the Mediterranean (southern Israel). *Carthamus persicus* is found in Iraq, Lebanon, and Syria. *Carthamus oxyacanthus* is endemic to Turkey, subtropical regions of western Iraq, Iran, North West India, throughout Kazakhstan, Turkmenistan, and Uzbekistan (Knowles and Ashri, 1995; Smith, 1996).

Data obtained from artificial crosses of safflower with other species can be used as an initial indicator to predict the potential for hybridization and introgression of a transgene into a weedy population. Hybridization is only the first step to introgression. For a hybridization event to occur, two species must be sympatric and flower synchronously. Four wild safflower relatives have been introduced in some areas of the New World (Hickman, 1993) (Fig. 3-1). Geographic locations (spatial) of these species have been documented in the U.S. and Canada (Fig. 3-1). *Carthamus oxyacanthus* has been naturalized in Oregon and Florida (Hickman, 1993) and is known from a single collection in California in 1978 (Keil, 2006). *Carthamus lanatus* has been introduced to Oregon and Floria and reported in Arizona. It has also been reported in Massachusetts, and New Jersey. *Carthamus lanatus* has been a weed in California since 1891 (Fuller, 1979). Kessler (1987) documented *C. lanatus* as a serious weed growing in an isolated area of Oklahoma. *Carthamus lanatus* and *C. leucocaulos* Sibth. et Sm. have been documented in Texas and collected from Argentina and Chile (Correll and Johnston, 1970; Hickman, 1993; Marticorena and Quezada, 1985). *Carthamus creticus* has been reported in California (Hickman, 1993; Hoover, 1970; Munz, 1968). Little information regarding the flowering times (temporal) in these localities has been reported.

Potential recipients of nuclear encoded transgenes from a cultivated PMP with safflower in the New World include safflower escapes from cultivation and four

naturalized wild relatives (*C. creticus*, *C. lanatus*, *C. leucocaulos*, and *C. oxyacanthus*). Of these wild species, only *C. oxyacanthus* and *C. creticus* have been shown to produce viable hybrid offspring when crossed with cultivated safflower (McPherson et al., 2004) (Fig. 3-1).

DOMESTICATION TRAITS IN *CARTHAMUS*

Safflower is considered one of humanities' oldest crops (Johnston et al., 2002; Knowles and Ashri, 1995; Smith, 1996). Domestication of this thistle species has resulted in a suite of traits that increased seed recovery at harvest, reduced seed dormancy, and increased yield and quality parameters, previously defined as the "domestication syndrome" (Harlan, 1992). Domestication traits have been compared between safflower and weedy relatives and their patterns of inheritance in hybrids used to infer the number of genes or loci involved and their dominant or recessive nature (Kotecha and Zimmerman, 1978; Zimmerman, 1972).

Shattering resistance is a well-established domestication trait that reduces seed loss and increases harvestability. The wild and weedy species *C. oxyacanthus*, *C. pericus*, and *C. palaestinus* have shattering capitulum (head) (Ashri and Efron, 1964; Imrie and Knowles, 1970; Knowles and Ashri, 1995; Smith, 1996). Nonshattering cultivated safflower lines were homozygous recessive (*sh*) for a single locus responsible for shattering, whereas, *C. persicus* and *C. palaestinus* are homozygous dominant for this locus (*Sh*) (Ashri and Efron, 1964; Imrie and Knowles, 1970).

The presence of a pappus (seed appendage for dispersal via water, wind, and adherence to animal fur) alters seed dispersal in the Asteraceae. Most of the achenes (seeds) of cultivated safflower lack a pappus and, when it is present, it is reduced (less

than the length of the achenes). The allele controlling the presence of a pappus in *Carthamus persicus* is dominant (*P_*) and character safflower is homozygous recessive for this locus (*pp*).

A single dominant gene (*Ro*) reduces the duration of the rosette stage in cultivated safflower. The presence of this gene reduces time to maturity. *Cathamus persicus* has a much delayed rosette appears stage (*ro*) (Imrie and Knowles, 1970). The delayed rosette stage of both *C. persicus* and *C. oxyacanthus* ensures that seeds are dispersed in the field after harvest of the cereal crops in which they commonly occur (Imrie and Knowles, 1970).

Reduced seed dormancy is a domestication trait of many crops that ensures synchronous germination and maturity and reduces volunteers in subsequent crops. However, complete loss of seed dormancy can lead to germination of mature seeds in the flower, prior to harvest, when conditions are humid (Evans, 1993). Cultivated safflower has a high germination rate suggesting that it exhibits low seed dormancy. Germination of seeds in mature heads prior to harvest after a heavy dew or rain has been identified as a production limitation for this crop (Zimmerman, 1972). Seed dormancy in safflower is controlled by many loci, the genes are not additive, and heritability was high (Kotecha and Zimmerman, 1978). The expected increase of dormancy under selection was approximately 10 to 25%.

It has been suggested that there is an association between lack of seed dormancy and some morphological traits. *Carthamus oxyacanthus* and *C. palaestinus* have pigmented (*w*) and mottled (*m*) seeds, which possess long- and short-term seed dormancy (Kotecha and Zimmerman, 1978).

The weed *Carthamus lanatus* has a high level of secondary seed dormancy (approximately 90%) and seeds persist in the soil for up to 10 years (Quinlivan and Pierce, 1969; Wright et al., 1980). Dormancy of these seeds can be broken by a combination of leaching and exposure to red light (600 to 680 nm), conditions which are often present when the soil is prepared for planting (Wright et al., 1980). Seeds of *C. oxyacanthus* have seed dormancy (10% or more are dormant), but scarification can sometimes improve germination, although cold treatments do not (Bassiri et al., 1975; Bassiri and Rouiiani, 1976). Scarification of white seeds reduced germination, whereas, it improved germination of seeds with pigmentation (Bassiri and Kheradnam, 1976), suggesting a genetic association of seed dormancy with seed pigments.

Safflower seeds with striped hulls (*ww mm*) have more seed dormancy than those with the typical white seed coloration (*W_M_*). Safflower typically has cotyledons with a green midvein, whereas, wild relatives such as *C. palaestinus* have purple midveins. Safflower cultivars with where cotyledons expressing green midveins have more seed dormancy than those with purple midveins (Bassiri and Kheradnam, 1976; Imrie and Knowles, 1970).

Domestication traits such as reduced shattering, lack of pappus and short duration of the rosette stage ensure that the majority of safflower seeds are harvested. Reduced seed dormancy ensures the seeds germinate when planted and are less likely to persist in the seed bank. Other traits associated with domesticated safflower include a restriction of branching to the upper portion of the stem (rather than flowering side shoots), a reduction of hairs on stamen filaments, and a reduction in the spine length on the leaves (Imrie and

Knowles, 1970). The genetic control of these traits and their functionality has not yet been studied in detail.

{{166 Berville,A. 2005; }}If a transgenic safflower were to hybridize with weedy relatives, recessive domestication traits may not be expressed. Hybrids may be more feral or invasive than cultivated safflower and could become problem weeds. The herbicide resistance trait may also confer an enhanced ability to survive in the agroecosystem. To reduce the risk of gene flow, transgenic safflower should be grown in areas free of wild relatives know to be cross-compatible.

FERALITY OF SAFFLOWER

Anecdotal reports suggest that safflower does not become established outside of agroecosystems (Smith, 1996). However, volunteer safflower has been documented in the U.S. from Arizona (Keil, 2006), California (Hickman, 1993; Keil, 2006; Munz, 1968), Colorado (Keil, 2006), Idaho (Keil, 2006), Illinois (Keil, 2006), Iowa (Keil, 2006; Rydberg, 1971), Illinois (Henry, 1992; Keil, 2006), Kansas (Gates, 1940; Keil, 2006; Rydberg, 1971), Massachusetts (Keil, 2006), Montana (Keil, 2006), Nebraska (Keil, 2006), New Mexico (Keil, 2006; Martin and Hutchins, 1981), North Dakota (Keil, 2006), Ohio (Keil, 2006; Vincent and Cusick, 1998) Oregon (Keil, 2006), Utah (Keil, 2006; Shaw, 1989) Washington (Keil, 2006) (Table 2). In Canada it has been reported in Alberta and British Columbia (Keil, 2006). It is not known how long these populations persist and whether they have become de-domesticated. Therefore, at least in some North American locations, PMP safflower has the potential to become established within the agroecosystem.

If PMP safflower is grown near existing feral populations, the PMP traits may move via pollen flow. Cross-pollination of safflower plants is facilitated predominantly by insects, but wind can also move pollen short distances (up to approximately 122 cm) between plants grown close together (Ashri and Rudich, 1965; Claassen, 1950). Bees and other flying insects contribute to gene flow among safflower and its wild relatives over relatively large distances (Claassen, 1950; Johnston et al., 2002). The frequency of outcrossing was partially under genetic control, ranging from, 0 to 100% among experimental cultivars (Claassen, 1950). Thus, each cultivated variety of safflower considered for the production of PMP should to be evaluated to determine the potential outcrossing rate.

INFORMATION REQUIRED TO IMPROVE OUR PREDICTION FOR PMP SAFFLOWER FERALITY

Prior to the release or growing of broad-scale PMP safflower under confined release conditions, considerable information is required to assess environmental biosafety impacts, including:

- The likelihood of volunteers surviving and perpetuating in the natural environment
- A quantification of gene flow from PMP safflower between feral populations and conventional varieties
- The risk of introgression of the PMP genes to wild/weedy populations
- The potential for persistence in the environment of PMP safflower/weed hybrids

CONCLUSIONS

Biosafety concerns associated with the production of PMP in safflower need to be addressed prior to release, including the potential for ferality. Domestication traits have been selected in safflower and include decreased shattering, seed dormancy, pappus length, and duration of the rosette stage. Most alleles controlling domestication traits are recessive. In the New World, two safflower relatives, *C. oxyacanthus* and *C. creticus*, are known to be cross-compatible with cultivated safflower. Hybrids between safflower and these wild relatives could serve as sinks for PMP traits and sources of feral traits. Thus, hybrids could facilitate introgression of PMP genes into conventional safflower or weedy relatives. Alternatively, hybrids could transfer feral traits to PMP safflower, which may enhance ferality. For this reason, PMP safflower should not be grown in the Mediterranean area where many cross-compatible species are now and where extensive cropping of safflower for human and animal consumption occurs. Further, regions in the North America where wild relatives or conventional safflower are grown should be avoided or appropriate isolation distances determined and maintained. Production of plant-made pharmaceuticals has great economic potential (Lacey et al., 1998; Moloney, 2000; Parmenter et al., 1995; Smith, 1996) and minor crops are platforms that may provide reduced risk to food and feed products. However, like all transgenic crops, risks and benefits must be evaluated on a case-by-case basis.

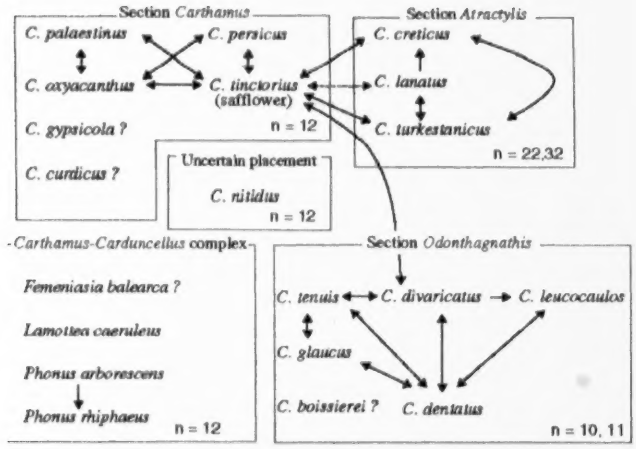

Figure 3-1. Artificial interspecific crosses that resulted in fertile progeny (see text for references). Solid lines indicate F_1 fertile hybrids with viable seed production. Dotted lines indicate hybridization occurred, but the F_1 hybrids could not be obtained without embryo rescue or treatments with colchicine. Arrows indicate the direction of the cross (male → female). Species shown in bold are naturalized in North America. Boxes indicate the taxa assigned to each section of *Carthamus sensu* (Lopez-Gonzalez, 1989) except the species that have since been reassigned to different genera within the complex. *Carthamus glaucus* contains several subspecies, which are all inter-fertile (Knowles and Schank, 1964). However, interspecific crosses with *C. glaucus* ssp. *glaucus* from different geographic regions are sterile. Crosses between *C. divaricatus* and safflower produced self-incompatible hybrids, which had low fertility when backcrossed with safflower. The symbol ? indicates taxa where crosses have not been reported. Documented locations of introduced species to the North America: *C. oxyacanthus*, California, Florida and Oregon; *C. creticus*, California, British Columbia, Nevada, Oregon and South Carolina; *C. lanatus*, Arizona, California, Florida, Massachusetts, New Jersey, Oklahoma, Oregon, and Texas; *C. leucocaulos*, California; *Carthamus tinctorius* (cultivated safflower), Alberta, Arizona, California, Colorado, Florida, Idaho, Illinois, Iowa, Kansas, Massachusetts, Montana, Nebraska, New Mexico, North Dakota, Ohio, Oklahoma, Oregon, Utah, Texas and Washington.

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Chapter 4: Outcrossing frequency of transgenic safflower (*Carthamus tinctorius* L.) intended for plant molecular farming

INTRODUCTION

Safflower is grown for its seed oil throughout the Mediterranean, Europe and in the Americas including the US and Chile. In the Canadian prairies, safflower is a minor crop grown for the non-food market with production limited to 320 to 810 ha annually (Muendel et al., 2004). Recently, safflower (*Carthamus tinctorius* L.; cv. 'Centennial') has been transformed for plant-molecular farming (PMF) using constructs encoding a seed targeted high-value protein and constitutive expressed *phosphinothricin acetyltransferase* (*pat*) to confer resistance to the broad-spectrum herbicide glufosinate (L-*phosphinothricin*). One concern with this new technology is outcrossing from transgenic to commodity safflower. The consequences of contamination of commodity crops via pollen-mediated gene flow can be serious, including halting the development of a crop as a PMF platform. For example, pollen movement from a small experiment of transgenic corn expressing a gene for a pig vaccine intended for PMF being developed by ProdiGene Inc. resulted in cross-fertilization of a field of corn intended for food/feed in Iowa. The neighbouring fields of 63 *ha.* of corn growing near the site were destroyed by USA government regulators (Ellstrand, 2003).

Crop species and varieties have different rates of outcrossing that are controlled genetically and influenced by the environment (for example see Beckie et al., 2003; Eastham and Sweet, 2002; Fritz and Lukaszewski, 1989; Hanson et al., 2005).

Outcrossing for both wind and insect pollinated species decreases exponentially with distance from the pollen source with the nearest neighbours receiving most of the pollen (Levin and Kerster, 1974). Various factors affect outcrossing among plant populations: pollinator effects including pollinator species and distance to other pollen sources; spatial and abiotic factors including distance to compatible crops, humidity, wind direction and velocity and geographic and vegetative barriers; crop species effects including the number and diversity of plant species attractive to pollinators in the area, ploidy level of the populations, shape, size and density of pollen donor and receptor plant populations, floral synchrony, floral and inflorescence position on the plant, pollen longevity, and cross-compatibility. Many of these factors interact, suggesting that predictions are difficult and require empirical measurements (Luna et al., 2001; Messeguer, 2003; Rognli et al., 2000). While complete containment of pollen and seed is not possible for any crop species to date (Levin and Kerster, 1974), management that incorporates spatial, temporal or vegetative barriers could minimize pollen-mediated gene flow between crops.

Safflower floral morphology and development influence the rate of self-pollination. Claassen (1950) documented that self-pollination rate varied for different safflower varieties (genotypes) and ranged from 9.3 to 81.5%. Bees have been observed combing pollen from the stigmatic hairs of safflower in the early morning, which facilitates pollen reaching the stigma and encourages plant-to-plant movement of pollen (Howard et al., 1915). For pollen delivered by an insect to result in outcrossing, however, it must out-compete the floret's own pollen.

Studies conducted in India using orange or red (dominant trait) safflower types as the pollen donor and white (recessive trait) types as the pollen recipient demonstrated that

safflower outcrossing decreases rapidly over relatively short distances (1 to 50 m) (Deokar and Patil, 1976; Howard et al., 1915; Kadam and Patankar, 1942). Howard et al. (1915) compared outcrossing rates among four varieties grown in close proximity and found the mean outcrossing rate to be 16.5% with a minimum and maximum value of 11.5 to 27%. Kadam and Patankar (1942) used several white-flowered plants together in close proximity with a plot of an orange floral type (dominant; variety not specified) at one end of the field. The outcrossing frequency from orange to white type when grown side by side was 10%; other white types separated by a few meters up to 13.7 m had mean outcrossing rates ranging from 1.5 to 2.3% (Kadam and Patankar, 1942). Deokar and Patil (1976) incorporated distance and directionality into their experimental design. The observed mean outcrossing frequency from one variety to another (N-62-8 and Nagpur-7) separated by 0.6 m was 0.8% with a range of 0.08 to 1.29%. Outcrossing was limited from 3 to 47.6 m from the pollen source, with a range of 0 to 0.12%. They did not observe a strong directional effect in outcrossing.

The only study of safflower outcrossing conducted in the USA also used corolla colour as a marker (Claassen, 1950). Using insect exclusion cages over safflower plants, they showed that wind did not move pollen beyond 1.2 m, suggesting that safflower outcrossing is primarily mediated by insects (Claassen, 1950). Outcrossing frequencies for different cultivated varieties grown in close proximity without insect exclusion ranged from 0 to 100%, with most between 0.5 to 40%, but higher oil-yielding safflower lines had outcrossing rates from 1 to 5%. The outcrossing rate of transgenic 'Centennial' safflower, a variety developed in the USA with high seed oil content and intended for

PMF in the Americas, was unknown. This cultivar readily produces seeds under greenhouse conditions when insects are excluded, suggesting it is highly self-pollinating.

This chapter of my thesis includes three experiments conducted to quantified the frequency of outcrossing from transgenic safflower to non-transgenic safflower under field conditions in three different environments at El Bosque (Santiago, Chile) in 2002, Westwold (British Columbia, Canada) in 2002 and Lethbridge (Alberta, Canada) in 2004. Transgenic and non-transgenic safflower (cv. 'Centennial') were used as pollen source and recipient, respectively, in these experiments. Outcrossing from transgenic to non-transgenic safflower produced a hemizygous seed resistant to the herbicide glufosinate. Seed harvested from the non-transgenic plants at various distances and directions from the transgenic pollen source were grown in subsequent years and screened for glufosinate resistance. Surviving plants were counted and the presence of the transgene confirmed at the protein and DNA level using molecular techniques. Pollen movement as a function of distance was modeled using regression analysis. Heterogeneity of outcrossing by direction was assessed with log-likelihood ratio test. To draw conclusions about samples that did not contain a transgenic seed, I conducted a power analysis using a binomial distribution to determine the minimum number of seeds (sample size) from outcrossing experiments to be screened to detect at least one or more transgenic seeds at different theoretical outcrossing frequencies and confidence thresholds.

RESULTS

Seeds harvested from non-transgenic recipient plots at different directions and distances from the transgenic pollen source were screened in the field with two applications of glufosinate; survivors were considered to be a product of outcrossing. The frequency of

outcrossing was calculated as the total number of safflower seedlings surviving glufosinate as a proportion of the number that emerged (Tabs. 4-1 to 4-3). Of the 1258 herbicide-resistant seedlings observed in the field, 302 were from El Bosque, 902 from Westwold and 54 from Lethbridge (Tabs. 4-1 to 4-3). A PCR analysis of 539 of the field survivors confirmed they all contain the transgene, *pat*; immunochromatography of 99 of the survivors confirmed all contain the transgenic protein, PAT.

Outcrossing frequency was modeled using an exponential decay function. Parameter estimates for intercept (a) and the rate of decline of outcrossing (b) were significant ($p < 0.0001$), indicating that the model was not over-parameterized. The rate of decline (b) was steepest at Lethbridge (Tabs. 4-1 to 4-4). The distance at which outcrossing frequency was reduced by 50% (O_{50}) ranged from 6.1 to 11.4 m; values for O_{90} ranged from 20.4 to 37.9 m (Tab. 4-4). Closest to the source, the frequency (percentage) of outcrossing was 0.48% at Chile, 1.67% at Westwold and 0.62% at Lethbridge (Tabs. 4-1, 4-2 and 4-3, respectively). Over all three experiments, the mean outcrossing frequency ranged from 0.0 to 0.86% in the first 10 m and from 0.0 to 0.54% at 10 to 20 m from the transgenic pollen source. At distances of 50 to 100 m from the transgenic pollen source, the mean outcrossing frequency ranged from 0.0024 to 0.03% (Tabs. 4-1 to 4-3). The highest rate of outcrossing was 1.67% at a mean distance of 3.0 m at the Westwold site (Tab. 4-2).

Outcrossing directionality was not uniform at all sites, suggesting wind direction or spatial aggregation of insect pollinators played a significant role in the pattern of safflower outcrossing. Several of the blocks for all of the outcrossing experiments were significantly heterogeneous, as determined from the maximum-likelihood ratio test (Tab.

4-5). Bi-weekly observations at each site indicated that flowering of the transgenic pollen source plants and the non-transgenic recipient plants was synchronous so it is unlikely that flowering time influenced outcrossing rates.

No field survivors were detected from all of the blocks at a mean distance of 23.3 and 300 m from the source at Chile and Westwold, respectively (Tabs. 4-1 and 4-2). At Lethbridge, survivors were not detected from all blocks at several distances from the source (Tab. 4-3). For samples where no transgenic seedlings were found, I employed binomial probabilities and sample sizes to conduct a power analysis to estimate the minimum detectable differences or limits of detection (Tab. 4-6).

Outcrossing was detected at all distances at the Chilean site except from the 18,840 seeds screened from samples taken at a mean distance of 22.6 m in eight directions from the transgenic pollen source (Tab. 4-1). From the power analysis results (Tab. 4-6), I would accept the null hypothesis that the frequency of transgenic seeds screened from these samples was equal to or greater than 0.00025 only 1% of the time. Thus, it is likely that the frequency of transgenic seeds at this distance was was less than 0.00025.

Outcrossing was detected at all distances for the Westwold site except from the 85,239 seeds screened from the single non-transgenic plot 300 m from the transgenic pollen source (Tab. 4-2). From the power analysis, I would accept the null hypothesis that the frequency of transgenic seeds screened from this sample was equal to or greater than 0.00005 only 2.5% of the time.

Outcrossing was detected at very few distances at the Lethbridge site. None of the samples screened from the Lethbridge site at mean distances of 2.75 to 22.25 m from the transgenic pollen source contained a glufosinate-resistant seed (Tab. 4-3). From the

power analysis (Tab. 4-6) and the range of seeds screened from each distance, 4012 to 16,962, I would accept the null hypothesis 2.5% of the time that the frequency of transgenic seeds in these samples would be equal to or greater than 0.001 and 0.00025, respectively. The samples taken at a mean distance of 46.25 m in eight directions from the transgenic pollen source had 45,726 seeds screened and no transgenic seeds were detected (Tab. 4-3). From the power analysis, I would accept the null hypothesis only 2.5% of the time that the frequency of transgenic seeds screened from this sample was equal to or greater than 0.0001.

DISCUSSION

Safflower outcrossing among plants within 1 to 3 m from the transgenic pollen source at all three sites ranged from 0 to 2%, which is similar to the outcrossing rates reported for high oil-yielding safflower lines in the USA (1 to 5%) (Claassen, 1950). Outcrossing declined steeply with distance, but was still detectable at very low frequencies at the farthest distances measured at all three sites. Outcrossing from transgenic safflower to non-transgenic plants declines rapidly and was reduced below 50% in the first 6 to 12 m and below 90% at distance of 20 to 40 m. At the most extreme distances at all of the outcrossing experiments, low amounts of outcrossing were detected ranging from 0.066 to 0.004% at 50 to 100 m from the transgenic pollen source. The frequency of outcrossing from transgenic to non-transgenic Centennial safflower at distances greater than 3 meters was 10 times lower than that reported for safflower with different floral colouration in India (Deokar and Patil, 1976; Howard et al., 1915; Kadam and Patankar, 1942).

The power of detection of a transgenic seed in a given sample and the ability to estimate the frequency of transgenic seeds in a seed sample are related to sample size and the "actual" frequency. The power of a statistical test is the probability of the rejection of the null hypothesis when it is actually false (Type II error). In this instance, the null hypothesis is "Transgenic seed from an outcrossing event are not present in the sample". However, because all of the seeds in each sample were not tested, one or more transgenic seeds may have been present and were not in the sub-sample examined. Thus, we used a conservative estimate of our ability to detect transgenic seeds in a sample using the power analysis. The power analysis, assuming a binomial distribution, allowed interpretation of zero values, which occurred during the screening of large numbers of seeds from the outcrossing experiments. The ability to change the alpha value (potential Type I error; rejection of null hypothesis when it is true) and incorporation of the various sample sizes from the screening process allowed a robust estimate of the probable transgenic seed frequency in each sample.

The frequency of outcrossing was as heterogeneous among blocks (replicates) as it was among the sites. The regions where the outcrossing experiments were conducted all had predominantly westerly winds; however, safflower flowers over a period of weeks and the wind direction varies considerably. The results of the likelihood ratio test did not indicate greater outcrossing on the leeward side of any of the experiments. Because wind does not facilitate significant safflower outcrossing beyond 1.2 m (Claassen, 1950), it is likely that the heterogeneity in outcrossing pattern observed at all three outcrossing sites was due to non-random pollen movement by insects.

Outcrossing rates were influenced by environment. Outcrossing differed among the three experiments (Tabs. 4-1 to 4-4), being the highest at Westwold and lowest at Lethbridge (Tabs. 4-2 and 4-3). The observed outcrossing frequency nearest the transgenic pollen source at Chile was more similar to Lethbridge than Westwold (Tabs. 4-1 to 4-4). However, it may also have been influenced by differences in experimental design, which differed in the size of the transgenic pollen source plot and shape and distribution of the non-transgenic pollen recipient plants. The Chilean and Lethbridge experiments had similar transgenic pollen source sizes and rates of outcrossing. The Westwold experiment had a pollen source three times greater in size than the others and an outcrossing rate four times greater closest to the source with a slower decline in outcrossing over distance as indicated by the O_{50} and O_{90} values (Tab. 4-4 and Figs. 4-1). The experiments at Chile and Westwold had barren zones (patches free of vegetation) between the transgenic source plot and the non-transgenic recipient plots, whereas the experiment at Lethbridge did not (Fig. 4-1). The lack of barren zones at Lethbridge may have reduced the rate of outcrossing observed relative to the other two sites. Barren zones between patches of insect-pollinated crop increased the distance of outcrossing in experiments conducted with *Brassica rapa* L. (syn. *B campestris* L.) and *B. napus* L. (Manasse, 1992; Morris et al., 1994, respectively). The experimental designs would also provide different edge shapes for pollinators. Previous research has shown pollinators entering large monoculture fields may settle first at the edge and then move inward, increasing outcrossing at the field margin (Ramsay, 2005). In addition, studies have shown pollinators depositing pollen from the first plant visited in a series to the next few visited plants, creating a pattern of outcrossing referred to as a paternity shadow

(Cresswell et al., 1995). If pollinators at the Chilean and Westwold sites followed these patterns of behaviour, cross-fertilization rates would depend on the direction they approached the experiment where they would perceive the edge to be. At the Lethbridge site, the edge would have consistently been 50 m from the transgenic pollen source plot (Fig. 4-1).

Pollen movement may have been influenced by diverse-pollinating insects. Butler et al. (1966) documented 40 species of non-parasitic bees and their relative abundance on safflower experiments and commercial fields in Arizona. Native bees made up 10 to 15% of pollinators on safflower field experiments and 8 to 13% at the edge of commercial fields. Only 61% of native bee species were common among the commercial fields and 24% were single specimens. Thus, pollinator diversity and movement patterns can vary greatly over short distances in similar agroecosystems.

The experiments presented here were designed to quantify outcrossing on a relatively small scale (50 to 100 m) and do not predict maximum distances of pollen movement by pollinators or the distance required to isolate fields of transgenic safflower from outcrossing with commodity safflower. Long-distance bee foraging has been documented up to 11.2 km from the hive on sweet clover pollen (Ramsay, 2005) and one of 2000 marked bees was found foraging 7.1 km from the hive on safflower (Gary et al., 1977).

A trap crop consisting of non-transgenic plants surrounding transgenic safflower may minimize long-distance outcrossing by insects. Currently in Canada, trap crops are used to reduce pollen movement from transgenic canola/oilseed rape field experiments (CFIA, Canadian Food Inspection Agency 2006). The efficacy of a trap crop to reduce outcrossing has been documented for transgenic canola (*B. napus* L.; Morris et al., 1994;

Staniland et al., 2000) and cotton (*Gossypium hirsutum* L.; Kareiva et al., 1994). The 0_{90} for the safflower experiment with the highest outcrossing (Westwold) was 37.9 m, suggesting a trap crop of this width around a transgenic safflower field could significantly reduce the risk of outcrossing to distant commodity safflower fields.

A trap crop in addition to isolation by distance of safflower intended for PMF from commodity safflower should provide acceptable levels of admixture, should acheivable thresholds be established. Currently, seed growers maintain certified and foundation level safflower seed purity with an isolation distance of 400 m from other safflower varieties in the USA and Canada (Anonymous, 2007a; Anonymous, 2007b; CFIA, 2005). The results presented here should aid industry and regulators to designate best management practices to mitigate outcrossing from transgenic safflower intended for PMF to commodity safflower.

MATERIALS AND METHODS

Source of plant materials

To assess the influence of distance and direction on outcrossing frequency from transgenic safflower intended for PMF to non-transgenic safflower under different environmental conditions, I conducted experiments at El Bosque, Chile and Westwold, BC in 2002 and Lethbridge, AB in 2004. Experiments were designed and implemented by different research groups and therefore varied at each site (Fig. 4-1). Seeds for both the transgenic and non-transgenic safflower were provided by SemBioSys Genetics Inc. (Calgary, Alberta, Canada). These seed lots were tested for cross-contamination between transgenic and non-transgenic safflower by screening in a greenhouse with glufosinate.

Both seed lots were found to be true to type (described below under herbicide resistance of transgenic plants).

Environmental biosafety compliance

For all of the transgenic field experiments, containment protocols were followed, as outlined by the federal governments of Canada and Chile. These measures included appropriate isolation distances between transgenic and commodity safflower crops and other crop species, triple containment of seed to and from the site, cleaning and inspection of all equipment used at the sites, and frequent monitoring and control of safflower volunteers in the experimental sites and designated perimeters during the growing season and in post-harvest years.

Outcrossing experimental design and implementation

El Bosque, Santiago, Chile

This experiment was planted in the municipality of El Bosque, in the province of Santiago, Chile, near the Catholic University at Pirque (32° 47′ 49.18″ S; 70 ° 40′ 01.43″ W; elevation 732 m), on September 14, 2002 in a wheel and spoke design (Fig. 4-1). The transgenic pollen source plot in the middle of the experiment was 10 x 11.2 m and consisted of 16 rows each 10 m in length. Four rows spaced 0.70 m apart of non-transgenic safflower were planted around the transgenic source plot for a total width of 2.8 m. Eight non-transgenic safflower plots (blocks or replicates) with four rows of safflower 2.8 m wide were planted radially from the transgenic source plot. In addition, four rows of non-transgenic safflower were planted around the experiment in a rectangle of 115 x 85 m. The nearest glufosinate-resistant safflower field was 573 m away from the

experiment, and the nearest non-GM safflower was *ca.* 1000 m away. Neighbouring crops included an alfalfa (*Medicago sativa* L.) field *ca.* 200 m to the north east, corn (*Zea mays* L.) *ca.* 200 m to the south east, sunflower (*Helianthus annuus* L.) *ca.* 700 m to the south-west and sugar beets (*Beta vulgaris* L.) *ca.* 50 m to the north.

The experiment was harvested on March 15, 2003. Seeds were hand-harvested at 16 distance intervals from the source plot in each of the eight radial plots. The first two samples nearest the pollen source were 2.8 m wide. Subsequent samples were 1.2 m wide. To reduce the amount of transgenic seed collected and transported to Canada for screening, areas between some samples were not harvested (Tab. 4-1). A total of 16 samples were harvested per radial block (replicate). In addition, samples were taken at the four outer corners, which were at the farthest distance from the transgenic source (Tab. 4-1).

Westwold, British Columbia, Canada

This experiment was planted at Westwold, BC (50° 28′ 04.66″ N; 119 ° 45′ 09.26″ W; elevation 616 m), on May 17, 2002 in a comb design to maximize the distance of the pollen recipient plants to the source within the available field dimensions (Fig. 4-1). The transgenic pollen source plot was 30 x 30 m and the non-transgenic safflower recipient plots were planted in four blocks extending from one side of the source plot. The recipient plots were 107 x 1.6 m in 16 rows. In addition, a plot of non-transgenic plants was 300 m from the experiment on the other side of an alfalfa field. The nearest glufosinate-resistant safflower was several kilometres away from the experiment. The farm site was surrounded by mixed boreal forest trees and its associated flora.

This experiment was harvested on October 16, 2002 with a WinterStieger 2001 Elite research combine in an east/west direction with each sample consisting of a 1.2 m wide swath. Harvesting began furthest from the source plot to reduce the chance of cross-contamination of samples from plots closer to the source. Commingling of the non-transgenic samples closest to the transgenic pollen source at the Westwold site during harvest was suspected and these samples were removed from the final analysis. The non-transgenic plot 300 m from the source was harvested separately after the harvester was cleaned and inspected.

Lethbridge, Alberta, Canada

This experiment was planted at Lethbridge AB (49° 41′ 51.91″ N; 112 ° 46′ 24.82″ W; elevation 909 m), on May 10, 2004 in a bulls-eye design. The transgenic source plot was 10 x 9.9 m consisting of 55 rows, located in the center of the experiment. The recipient non-transgenic safflower was planted 50 m around the transgenic pollen source plot with row spacing of 18 cm. Once the safflower plants had bolted, the outside of the non-transgenic recipient plot was mowed into a circle that resulted in the outermost plants located in a 50 m perimeter around the transgenic pollen source. The closest transgenic and non-transgenic safflower was over 50 km from the experiment. The experiment was conducted on a research farm with a diversity of crop species grown, including barley (*Hordeum vulgare* L.) to the east, west and north for over 50 m and peas (*Pisum sativum* L.) to the south for over 50 m.

Harvest of the Lethbridge experiment was conducted on November 3, 2004. Prior to harvest, eight wedges were removed (*ca.* 1/3 of the site area) between the blocks intended for harvest (Fig. 4-1). The area removed between blocks had an outside arc distance of

14.4 m. The remaining area comprised eight blocks with an outside arc distance of 28.8 m and an inside arc distance of 2.5 m. Seeds were harvested in 1.2 m wide swaths from each block with a WinterStieger 2001 Elite research combine and each swath was considered a plot. Harvesting began distal to the source and continued inward to reduce the chance of cross-contamination of closer plots with those further away.

Herbicide resistance of transgenic plants

A dose-response experiment with the herbicide glufosinate and homozygous transgenic and non-transgenic safflower plants was conducted in the greenhouse to determine a discriminating dose (Beckie et al., 2000). Experiments were conducted in the greenhouse at the University of Alberta, Edmonton, Alberta from 2004 to 2006.

Transgenic and non-transgenic seeds were planted in trays with six cells each (1 L per cell) containing soil-less vermiculite-peat mixture (Metro-Mix 290, The Scott's Company, 14111 Scottslawn Rd., Marysville, Ohio 43041). Twelve seeds per cell were planted and thinned to eight plants after emergence. Plants were exposed to natural light supplemented for 16 h by 400 W high pressure sodium, high intensity discharge bulbs and maintained at 21/18 C° day/night temperature. Plants were watered as required and fertilized biweekly with complete 20-20-20 plus at a concentration of 200 ppm.

Glufosinate was applied to paired cells of transgenic and non-transgenic plants at 0, 0.25, 0.5, 1, 1.5 and 2 times the recommended field rate (400 g ai ha^{-1}) (Ali, 2003). Each herbicide dose-response experiment had three replicates arranged in a randomized complete block design and was repeated three times. Herbicides were applied (using a custom built track sprayer with a Billericay Air Bubble Jet 110015 nozzle tip calibrated to deliver 100 L ha^{-1} at 200 KPa) when safflower had two to six true leaves. After

herbicide application, trays were returned to the greenhouse and irrigated from above as required, but not before 24 h after application. Twenty-one days after herbicide application, plants were counted and cut at the soil surface, dried in paper bags at 60 °C for five days and dry weight measured.

Transgenic plants did not show signs of herbicide damage at rates as high as 800 g ai ha^{-1} with a water volume of 100 L ha^{-1} when the plants were at the two- to six-leaf stage, but occasionally a non-transgenic plant would survive. Further experiments were conducted with glufosinate applied at a rate of 800 g ai ha^{-1} and an increased water volume of 200 L ha^{-1} applied twice at a 4- to 7-day interval between applications. None of the 738 non-transgenic plants survived and none of the 738 transgenic plants showed no signs of herbicide injury.

Because pollen-mediated gene flow in the outcrossing experiments resulted in hemizygous seed, a second experiment was conducted to ensure the high rate of glufosinate used on the homozygous plants would not damage plants with a single copy of the herbicide resistance gene. Hand-pollination of homozygous transgenic 'Centennial' safflower plants with a non-transgenic 'S-317' safflower (and the reciprocal) was performed to generate hemizygous seeds. These seeds were planted in the greenhouse and treated as described above, except they were sprayed with glufosinate at 800 g ai ha^{-1} with double the previous water volume (200 L ha^{-1}). All of the plants survived and did not show signs of herbicide injury. The F_1 seeds harvested from the surviving hemizygous parents were planted and sprayed as above. Chi-square analysis of the F_1 plants with and without herbicide damage fit the predicted three to one ratio, confirming the parents were hemizygous and not the product of self-fertilization. These results were

used to establish the herbicide rate and water volume to discriminate between transgenic and non-transgenic safflower in the field screening of the seed samples harvested from the outcrossing experiments.

Seed screening

Seeds from all experiments were cleaned and stored at 10 °C until field screening was performed. The seed from the Chilean site was grown in 2004 and from the Westwold and Lethbridge sites in 2005 on land in Edmonton, Alberta, never utilized for safflower cultivation. Seeding rates were adjusted for a target of 250 plants m^{-2} based on seed germinability and kernel weight. An early frost at the Lethbridge site reduced seed quality; the germination percentage ranged from 4 to 40% and the 1000-seed weight ranged from 13 to 29 g. Linear regression of germination percentage and 1000-seed weight for a subset of the samples was conducted ($R^2 = 0.86$). Seeding rates for the samples from the Lethbridge experiment were based on the slope and intercept values estimated from the regression analysis and the 1000-seed weights from all the plots (distances and directions). On some occasions when germination percentage were extremely low, the maximum number of seeds that could be planted with the seeding equipment still did not provide the target density of 250 plants m^{-2}.

Prior to planting samples from the Chilean outcrossing experiments in 2004, the site was first tilled and glyphosate was applied at a rate of 1.5 L ha^{-1}. Prior to planting samples from the Westwold and Lethbridge outcrossing experiments in 2005, the site was first tilled, packed with a spiral packer and glyphosate applied at 810 g ai ha^{-1}. Seeds from the Westwold and Lethbridge sites were treated with Helix Xtra ® (an insecticide-fungicide mixture of thiamethoxam, difenoconazole, mefenoxam, and fludioxonil) as

recommended for canola to increase seedling emergence and survival. Previous germination experiments with Helix Xtra ® have shown that it does not inhibit germination (unpublished data). Each plot was planted with a seed sample from an outcrossing experiment to a depth of 1.3 to 3.8 cm and consisted of six rows 7 m long spaced 20.3 cm apart with a 1 m alley between plots. Fertilizer (0-45-0) was placed with the seed at a rate of 1.8 g m^{-1} of row. Planting was performed with a reduced-disturbance Fabro air seeder equipped with atom jet double shoot openers. After seeding each plot, hoses and openers were checked to ensure all seeds were cleared. Several control plots of non-transgenic and transgenic safflower were randomly positioned among the outcrossing- sample plots to ensure the transgene was functional and verify herbicide efficacy and coverage.

When safflower seedlings were at the four- to six-leaf stage, plant number per plot was estimated based on three, 0.25 m^2 quadrats per plot. Following emergence counts, the seedlings were sprayed with glufosinate at 800 g ai ha^{-1} using low drift Billericay Air Bubblejet nozzle tips that delivered a water volume of 200 L ha^{-1}. The application was repeated 4 to 7 d later to ensure non-transgenic plants did not survive because of a spray miss or shading from other plants. A week later, the surviving safflower plants were counted. The frequency of outcrossing was calculated as the number of survivors following herbicide application as a proportion of the number of emerged seedlings.

To reduce the land base required to screen samples, every second sample from the Westwold experiment was combined, thereby increasing the effective range of the distance from the transgenic pollen source (Tab. 4-2). In addition, to increase the number of seedlings screened and reduce the area of land dedicated to transgenic confinement,

seed from the Westwold and Lethbridge experiments were planted three times over the growing season. After the surviving plants were counted and sampled, the field area was treated with 810 g ai ha^{-1} glyphosate to kill all remaining transgenic safflower plants and any weeds. Seed from the same samples were planted into the same plots to ensure any volunteers from prior screening tests did not confound the results.

Molecular confirmation of field survivors

A leaf from plants surviving both applications of glufosinate in the field were harvested and frozen at -20°C. A subset of these samples were confirmed as transgenic using commercially available immunochromatographic lateral flow test strips (Strategic Diagnostic Inc.®) and event-specific end-point PCR (Tab. 4-7). Immunochromatography was carried out on *ca.* 0.25 cm^2 leaf tissue ground in the supplied kit buffer. After 5 minutes, the lateral flow test strips were inserted and allowed to develop for another 5 minutes.

For the PCR confirmation of field survivors, total genomic DNA was extracted from *ca.* 0.25 cm^2 leaf tissue using a CTAB-based protocol described by Doyle and Doyle (1987). Multiplex PCR was conducted with primers specific to a non-coding region of the safflower genome to indicate the reaction was working and primers specific to the herbicide resistance gene (*pat*) to confirm the presence of the transgene. The primers JCH1 and JCH4 were specific to the non-coding region and produced a 900 bp product; the primers JCH5 and JCH6 were specific to *pat* and produced a 350-bp product (Tab. 4-7). Each PCR was performed in 20-µl volumes using 10 to 40 ng of DNA template, 0.5 mM of each primer, 500 mM KCl, 100 mM Tris-HCl (pH 8.4), 3.0 mM $MgCl_2$, 0.25 mM each dNTP and 2 units of *Taq* DNA polymerase. Cycling conditions for amplification

consisted of 95 °C for 10 min, followed by 35 cycles of 95 °C for 20 s, 59 °C for 30 s and 72 °C for 45 s, followed by a final extension of 5 min at 72 °C. Ten percent of each PCR reactions were loaded into 1.5% agarose gels; electrophoresis was conducted to separate the products and then visualized using ethidium bromide under UV light.

Statistical analysis

Statistical analyses were conducted separately for each experiment (El Bosque, Westwold, and Lethbridge). Outcrossing frequency (f) was calculated as the ratio of glufosinate survivor(s) to estimated total seedlings emerged (n_o). A 95% confidence interval for the mean frequency of outcrossing at each distance was calculated, assuming a binomial distribution as described by Zar (1999; pp. 527-528) (Tabs. 4-1 to 4-3). This method is error-prone when a zero value is observed (the number of survivors in this study), thus an alternative method was used when this occurred, as described by Blyth (1986) in Zar (1999; pp. 528) (Tabs. 4-1 to 4-3).

Mean frequency of outcrossing (f) at each mean distance were subjected to regression analysis using a nonlinear regression mixed model (PROC NLMIXED) with Statistical Analysis Software (SAS, 2007). The binomial distribution (~ binomial (n_o, f)) was employed to approximate the dependent variable. The data were fit to equation (1), an exponential decay function (Hanson et al., 2005)

$$p = ae^{-bd} \qquad (1)$$

where p is the predicted outcrossing frequency, a is the intercept, b is a curve parameter (rate of decline), and d is the mean distance (m) from the edge of the source plot. Model fit was evaluated by the significance of the parameter estimates and visual examination of

the residual structure (Hanson et al., 2005). The standard error and 95% confidence interval were calculated for each parameter estimate.

Using the regression estimation of equation 1, I estimated the distance where outcrossing was reduced by both 50 and 90% (O_{50} and O_{90})

$$O_{50} = \frac{\ln 0.5 * a - \ln a}{-b} \qquad (2)$$

$$O_{90} = \frac{\ln 0.01 * a - \ln a}{-b} \qquad (3)$$

where a and b are intercept and slope, respectively.

To determine if there was heterogeneity of outcrossing among the blocks for each experiment, a log-likelihood ratio test or G-test using the chi-square distribution was conducted using the -2 log-likelihood score provided by SAS for regression analysis (as above) with outcrossing data sets partitioned by blocks compared to a data set containing all of the partitions (Zar, 1999; pp. 473-475) (Tab. 4-6).

In addition, a power analysis using binomial probabilities was conducted to determine the sample size required to detect at least one transgenic seed for samples with different theoretical frequency (transgenic seed content) and three different alpha values (Zar, 1999; pp. 539-542) (Tab. 4-6). The minimum sample size to detect at least one transgenic seed at a given frequency and alpha value was derived from the following formula

$$n_p \geq \frac{\ln \alpha}{\ln(1-p)} \qquad (4)$$

where n_p is the minimum sample size required to detect one or more transgenic seeds with a set value of α for a sample containing a theoretical frequency of transgenic seeds

(p). The null hypothesis that the frequency is equal to or greater than (p) can be rejected when zero transgenic seeds were found in a sample of seeds (n_p) with a confidence value related to the type I error alpha value by 1-α.

Table 4-1. Harvest distances and field screening results for the outcrossing experiment at El Bosque, Santiago, Chile.

Distance (m)[1]			Emergence[4]		Survivors	Observed outcrossing	95% confidence interval[5]	
Mean[1]	Minimum	Maximum	Mean	Total (n_o)	Total	%	Lower	Upper
0.70	0.00	1.40	2,313	18,504	89	0.48098	0.38760	0.59433
2.10	1.40	2.80	2,301	18,404	27	0.14671	0.09643	0.21377
3.40	2.80	4.00	2,645	21,156	22	0.10399	0.06501	0.15755
4.60	4.00	5.20	2,638	21,100	21	0.09953	0.06146	0.15215
5.80	5.20	6.40	2,831	22,648	28	0.12363	0.08201	0.17891
7.00	6.40	7.60	2,583	20,660	7	0.03388	0.01361	0.06967
8.20	7.60	8.80	2,591	20,728	20	0.09649	0.05885	0.14885
9.40	8.80	10.00	2,469	17,284	8	0.04628	0.01995	0.09108
Unharvested (1 m)[2]								
11.60	11.00	12.20	2,765	22,116	22	0.09947	0.06219	0.15072
12.80	12.20	13.40	2,981	23,844	4	0.01677	0.00457	0.04298
Unharvested (2 m)[2]								
16.00	15.40	16.60	2,239	17,912	10	0.00558	0.02672	0.10251
17.20	16.60	17.80	1,983	15,864	9	0.05673	0.02591	0.10774
Unharvested (3 m)[2]								
21.40	20.80	22.00	2,224	17,792	20	0.11241	0.06857	0.17340
22.60	22.00	23.20	2,355	18,840	0	0.00000	0.00000	0.02326
Unharvested (4 m)[2]								
27.80	27.20	28.40	2,301	18,408	10	0.05432	0.02600	0.09975
29.00	28.40	29.60	2,180	15,260	2	0.01311	0.00159	0.04736
Corner samples[3]								
52.06	50.66	53.46	2,816	5,632	1	0.01775	0.00045	0.13089

60.47	59.07	61.87	3,324	6,648	2	0.03008	0.00364	0.10867
Total	-	-	45,536	322,800	302	-	-	-
Mean	-	-	2,530	17,933	17	-	-	-

[1] Mean distance was used in all analyses. The last four samples were taken from the most distant corners of the rectangular- shaped experiment.

[2] To reduce the amount of transgenic material to be transported back to Canada for screening, harvested material in the area between some samples was not retained.

[3] The corner samples were from the four corners of the rectangular-shaped non-transgenic portion of the experiment (Fig. 4-1).

[4] The mean and total number of plants that emerged during the field screening with glufosinate for each distance sampled. Seed from three plots in block 8 were not screened (808 at 13.2 m, 812 at 23.0 m and 816 at 36.8 m from the source plot).

[5] The 95% confidence interval using a relationship between F and binomial distributions with a correction for the lower interval value when the number of survivors was zero as outlined in Zar (1999); pp. 528).

Table 4-2. Harvest distances and field screening results for the outcrossing experiment at Westwold, BC, Canada.

Distance (m)[1]			Emergence[2]		Survivors	Observed outcrossing	95% confidence interval[3]	
Mean	Minimum	Maximum	Mean	Total (n_o)	Total	%	Lower	Upper
0.75	0.00	1.50	-	-	-	-	-	-
3.00	1.50	4.50	3,129	12,514	209	1.67011	1.44739	1.93467
6.00	4.50	7.50	2,427	9,707	154	1.58654	1.34945	1.87555
9.00	7.50	10.50	2,520	10,081	87	0.86301	0.69374	1.08351
12.00	10.50	13.50	2,804	11,215	106	0.94517	0.77346	1.16426
15.00	13.50	16.50	2,569	10,274	69	0.67159	0.49719	0.85012
18.00	16.50	19.50	2,166	8,663	47	0.54252	0.39885	0.72119
21.00	19.50	22.50	2,020	8,081	21	0.25987	0.14647	0.38827
24.00	22.50	25.50	3,039	12,157	33	0.27145	0.18658	0.38162
27.00	25.50	28.50	4,151	16,606	17	0.10237	0.05961	0.16422
30.00	28.50	31.50	3,599	14,396	15	0.10420	0.05823	0.17238
33.00	31.50	34.50	2,770	11,081	10	0.09025	0.04320	0.16566
36.00	34.50	37.50	1,497	5,989	4	0.06679	0.01820	0.17096
39.00	37.50	40.50	1,523	6,093	10	0.16413	0.07858	0.30109
42.00	40.50	43.50	2,539	10,155	11	0.10832	0.05419	0.19364
45.00	43.50	46.50	2,191	8,766	13	0.14830	0.07893	0.25367
48.00	46.50	49.50	2,188	4,376	1	0.02285	0.00058	0.12737
51.00	49.50	52.50	1,864	7,455	3	0.04024	0.00830	0.11742
54.00	52.50	55.50	3,326	13,306	16	0.12025	0.06859	0.19584
57.00	55.50	58.50	3,745	14,978	6	0.04006	0.01473	0.08735

60.00	58.50	61.50	2,789	11,155	11	0.09861	0.00049	0.17628
63.00	61.50	64.50	2,199	8,796	9	0.10232	0.04674	0.19423
66.00	64.50	67.50	2,875	11,499	8	0.06957	0.03000	0.13688
69.00	67.50	70.50	3,185	12,738	6	0.04710	0.01732	0.10270
72.00	70.50	73.50	3,860	15,441	9	0.05829	0.02662	0.11068
75.00	73.50	76.50	4,521	18,084	3	0.01659	0.00342	0.04842
78.00	76.50	79.50	4,114	16,457	5	0.03038	0.00987	0.07070
81.00	79.50	82.50	5,111	20,444	4	0.01956	0.00533	0.05012
84.00	82.50	85.50	4,838	19,354	6	0.03100	0.01140	0.06761
87.00	85.50	88.50	4,984	19,936	6	0.03010	0.01107	0.06564
91.75	88.50	95.00	5,066	20,265	1	0.00493	0.00012	0.02753
101.00	95.00	107.00	4,678	18,711	2	0.01069	0.00129	0.03863
Total	-	-	98,287	388,770	902	-	-	-
Mean	-	-	3,171	12,541	29	-	-	-
300.00[4]	300.00	300.00	85,239	85,239	0	0.00000	0.00000	0.00514

[1] Mean distance was used in all analyses.

[2] The total and mean number of plants that emerged during the field screening with glufosinate for each distance sampled. Seed from three samples in blocks 2 and 3 were not screened (mean distance from the source of 45 m). Data for samples collected from the distance closest to the source (mean distance from the source 0.75 m) were excluded due to admixture during harvest.

[3] The 95% confidence interval using a relationship between F and binomial distributions with a correction for the lower interval value when the number of survivors was zero as outlined in (Zar, 1999); pp. 528).

[4] The single non-transgenic plot grown 300 m from the pollen source. These data were not included in the regression analysis but were screened in the field and are thus treated separately here.

Table 4-3. Harvest distances and field screening results for the outcrossing experiment at Lethbridge, AB, Canada.

Distance (m)[1]			Emergence[2]		Survivors	Observed outcrossing	95% confidence interval[3]	
Mean	Minimum	Maximum	Mean	Total (n_o)	Total	%	Lower	Upper
0.25	0.00	0.50	1,153	5,764	36	0.6245374	0.4368768	0.8631839
1.25	0.50	2.00	765	6,123	1	0.0163328	0.0004135	0.0910686
2.75	2.00	3.50	676	5,406	0	0.0000000	0.0000000	0.0810277
4.25	3.50	5.00	747	5,973	0	0.0000000	0.0000000	0.0733329
5.75	5.00	6.50	601	4,805	0	0.0000000	0.0000000	0.0911607
7.25	6.50	8.00	665	5,316	0	0.0000000	0.0000000	0.0823928
8.75	8.00	9.50	768	6,141	0	0.0000000	0.0000000	0.0713361
10.25	9.50	11.00	502	4,012	0	0.0000000	0.0000000	0.1091597
11.75	11.00	12.50	885	7,079	0	0.0000000	0.0000000	0.0618785
13.25	12.50	14.00	876	7,006	0	0.0000000	0.0000000	0.0625296
14.75	14.00	15.50	1,414	11,312	0	0.0000000	0.0000000	0.0387320
16.25	15.50	17.00	1,641	13,126	0	0.0000000	0.0000000	0.0333777
17.75	17.00	18.50	1,924	15,390	0	0.0000000	0.0000000	0.0284685
19.25	18.50	20.00	2,053	16,421	0	0.0000000	0.0000000	0.0266824
20.75	20.00	21.50	2,120	16,962	0	0.0000000	0.0000000	0.0258305
22.25	21.50	23.00	1,736	13,885	0	0.0000000	0.0000000	0.0315543
23.75	23.00	24.50	1,938	15,501	1	0.0064513	0.0001633	0.0359875
25.25	24.50	26.00	1,919	15,353	0	0.0000000	0.0000000	0.0285386
26.75	26.00	27.50	2,311	18,490	1	0.0054085	0.0001369	0.0301718
28.25	27.50	29.00	2,811	22,490	1	0.0044465	0.0001126	0.0248064
29.75	29.00	30.50	2,564	20,510	0	0.0000000	0.0000000	0.0213625

31.25	30.50	32.00	2,561	20,489	0	0.0000000	0.0000000	0.0213854
32.75	32.00	33.50	2,813	22,506	1	0.0044433	0.0001125	0.0247888
34.25	33.50	35.00	3,523	28,187	0	0.0000000	0.0000000	0.0155449
35.75	35.00	36.50	2,272	18,177	0	0.0000000	0.0000000	0.0241047
37.25	36.50	38.00	3,978	31,826	0	0.0000000	0.0000000	0.0137677
38.75	38.00	39.50	3,599	28,791	1	0.0034733	0.0000879	0.0193777
40.25	39.50	41.00	3,235	25,879	7	0.0270485	0.0108642	0.0556267
41.75	41.00	42.50	3,237	25,894	0	0.0000000	0.0000000	0.0169212
43.25	42.50	44.00	3,317	26,537	0	0.0000000	0.0000000	0.0165118
44.75	44.00	45.50	4,245	33,958	3	0.0088343	0.0018216	0.0257919
46.25	45.50	47.00	5,716	45,726	0	0.0000000	0.0000000	0.0095828
47.75	47.00	48.50	4,402	35,213	1	0.0028399	0.0000719	0.0158444
49.25	48.50	50.00	5,145	41,156	1	0.0024298	0.0000615	0.0135566
Total	-	-	78,108	621,404	54	-	-	-
Mean	-	-	2,297	18,277	2	-	-	-

[1] Mean distance was used in all analyses.

[2] The total and mean number of plants that emerged during the field screening with glufosinate for each distance sampled. Samples from the distances closest to the source for blocks 2, 4, 6 and 8 were not harvested to avoid potential admixture with the transgenic source plot.

[3] The 95% confidence interval using a relationship between F and binomial distributions with a correction for the lower interval value when the number of survivors was zero as outlined in (Zar, 1999) ; pp. 528)..

Table 4-4. Parameter estimates and O_{50} and O_{90} values with their respective standard errors and confidence intervals from the regression analysis and equation 1, 2 and 3. [1]

Experiment	Parameter[2]	Estimate[1]	Standard error	Df[3]	95% confidence interval Lower	Upper
Chile	*a*	0.0025	0.0002	129	0.0020	0.0030
	b	0.0757	0.0078	129	0.0603	0.0912
	O_{50}	9.1512	0.9421	129	7.2873	11.0151
	O_{90}	30.3996	3.1295	129	24.2077	36.5914
Westwold	*a*	0.0168	0.0008	124	0.0152	0.0184
	b	0.0607	0.0020	124	0.0566	0.0647
	O_{50}	11.4267	0.3838	124	10.6669	12.1864
	O_{90}	37.9586	1.2751	124	35.4348	40.4823
Lethbridge	*a*	0.0011	0.0002	269	0.0007	0.0015
	b	0.1126	0.0118	269	0.0893	0.1360
	O_{50}	6.1534	0.6469	269	4.8798	7.4270
	O_{90}	20.4412	2.1490	269	16.2103	24.6721

[1]Estimates of the parameters for intercept (*a*), slope (*b*) and the estimates of the distance where outcrossing was reduced to 50 and 90% (see text for regression equation). All predictions were significant ($p < 0.0001$).
[2]Parameters a and b were estimated from the regression analysis with equation 1. The distance that outcrossing was reduced by 50 and 90% were estimated using equation 2 and 3, respectively.
[3]Degrees of freedom.

Table 4-5. Outcrossing directionality: log-likelihood ratio test among blocks for each experiment.

Experiment	Block (s)	Direction[1]	Combined value[3]	df[7]	Partitioned value[4]	df[7]	X^2 value[5]	df	Significance[6]
Chile	All	All	693.2	16	571.3	2	121.9	14	****
	1 vs. 5	N and S	234.8	4	221.0	2	13.8	2	***
	2 vs. 6	NE and SW	128.9	4	125.4	2	3.5	2	*ns*
	3 vs. 7	E and W	143.5	4	95.0	2	48.5	2	****
	4 vs. 8	NW and SE	145.8	4	129.9	2	15.9	2	****
Westwold	All	All	722.7	8	687.0	2	35.7	6	****
	1 vs. 2	N and S[2]	301.8	4	297.5	2	4.3	2	*ns*
	2 vs. 3	N and S[2]	307.4	4	289.8	2	17.6	2	****
	3 vs. 4	N and S[2]	398.5	4	389.5	2	9.0	2	*
Lethbridge	All (less 1, 3 and 8)[8]	Subset of all	312.4	10	149.2	2	163.2	8	****
	2 vs. 6	NE and SW	21.3	4	19.9	2	1.4	2	*ns*

[1] Direction from the transgenic pollen source where 'N' is north, 'S' is south, 'W' is west and 'E' is east.

[2] The Westwold blocks were in a North-South direction relative to the transgenic source plot, but are east and west of one another.

[3] The overall value is the -2 log-likelihood value calculated by SAS (2007) for the regression of the combined data set for the blocks being compared.

[4] The individual sum value is the sum of the -2 log-likelihood values calculated by SAS (2007) for each individual regression of the partitioned data sets for the blocks being compared.

[5] The chi-square value was calculated as twice the value of the log-likelihood ratio of the sum of the partial data sets and the combined data set.

[6] Significance are given for alpha $\leq$ 0.05, 0.01, 0.005 and 0.001 corresponding to *, **, *** and ****, respectively. When the value was not significant it was designated as *ns*.

[7] Degrees of freedom.

[8] At the Lethbridge site the blocks 3 and 8 were excluded from the log-likelihood ratio test because the observed outcrossing frequency was zero at most distances for three and all zeros for 8 and the program could not converge during the analysis. When block 1 was analyzed by itself the program gave a warning and did not calculate the standard error and confidence intervals. This may have occurred because the only survivors observed were at the distal portion (farthest from the transgenic source plot) and for this reason this block was excluded from the log-likelihood ratio tests.

Table 4-6. Power analysis assuming a binomial distribution and using equation 2 (see text) to determine the minimums number of seeds to screen to detect at least one transgenic seed for different levels of outcrossing and three alpha values.

Null hypothesis[1]	Alpha value (α)			
Frequency of p(X)	0.05 (5%)	0.025 (2.5%)	0.01 (1%)	0.005 (0.05%)
	Minimum sample size (n_p)[2]			
0.01	299	368	459	528
0.005	598	736	919	1,058
0.0025	1,197	1,474	1,840	2,117
0.001	2,995	3,688	4,603	5,296
0.0005	5,990	7,376	9,209	10,594
0.00025	11,982	14,754	18,419	21,191
0.0001	29,956	36,887	46,050	52,981
0.00005	59,914	73,776	92,102	105,964
0.000025	119,828	147,554	184,205	211,931
0.00001	299,572	368,887	460,515	529,830
0.000005	599,145	737,775	921,032	1,059,661
0.0000025	1,198,292	1,475,550	1,842,066	2,119,325

[1] The theoretical value of outcrossing (p).

[2] The minimum value of n_p is the sample size required to detect one or more transgenic seeds given a theoretical frequency of transgenic seeds (p) and different values of alpha. Values for minimum sample size were rounded upward to ensure they are within the bound set by the alpha value. The null hypothesis that the frequency is $X \geq p$ is rejected at a given percentage (alpha value) of the time, when no transgenic seeds were found in a sample size of n_p or greater.

Table 4-7. Primers used to confirm safflower plants that survived glufosinate field screening at Edmonton, Alberta, Canada.

DNA sequence description	Forward primer name and sequence	Reverse primer name and sequence	Annealing temperature (°C)	Product size (bp)
Non-coding region from safflower nuclear genome	JCH1 CAC ACT AAG CCA CTC CAA CC	JCH4 TTG ACA ACT CCA ATC CCT GC	59	900
Portion of the transgene *pat* (*phosphinothricin acetyltransferase*)	JCH5 GAT CTG GGT AAC TGG TCT AAC TGG	JCH6 GTT GCA AGA TAG ATA CCC TTG GTT	59	350

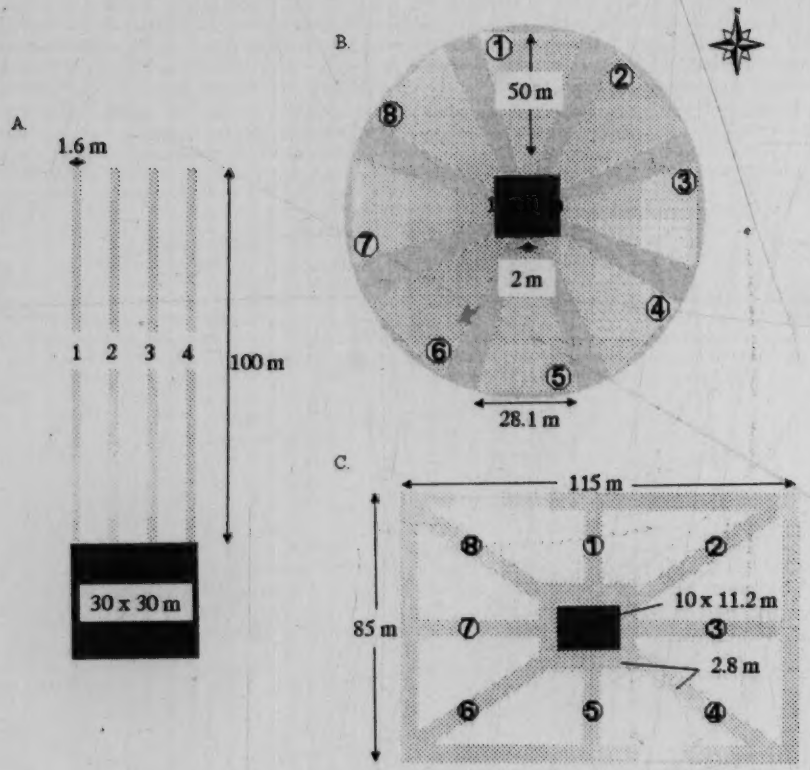

Figure 4-1. Experiment designs showing the spatial arrangement of the transgenic pollen source (darkest colour), non-transgenic recipient plants (light and dark grey) and the area free of vegetation (white). (A) Westwold, British Columbia, (B) Lethbridge, Alberta, and (C) El Bosque, Chile. The light grey area was sampled and retained, whereas the dark grey area was not retained at harvest; numbers indicate blocks or replicates (numbered).

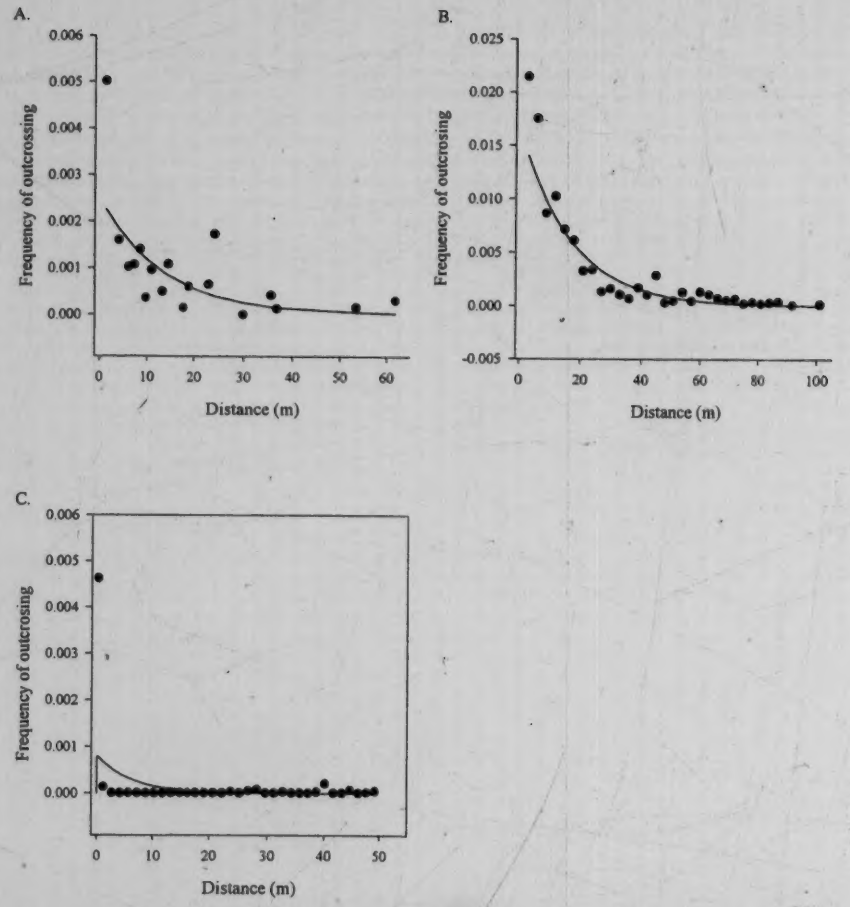

Figure 4-2. Percent outcrossing with increasing distance from the transgenic pollen source at (A) El Bosque Chile in 2002, (B) Westwold in 2002, (C) Lethbridge in 2004 (see Tab. 4-4 for regression equation and parameter estimates).

Appendix

Table 4-A1. Results of the regression analyses of the outcrossing experiment at El Bosque, Santiago, Chile with the data from each block.

Experiment site	Block	Parameter	Estimate[1]	Standard error[2]	Df[3]	95% confidence interval[4] Lower	Upper
Chile	1	*a*	0.0034	0.0010	16	0.0013	0.0055
		b	0.1023	0.0258	16	0.0476	0.1570
	2	*a*	0.0023	0.0007	17	0.0008	0.0038
		b	0.1058	0.0286	17	0.0454	0.1662
	3	*a*	0.0166	0.0034	16	0.0092	0.0239
		b	0.2744	0.0342	16	0.2018	0.3469
	4	*a*	0.0006	0.0002	17	0.0001	0.0012
		b	0.0235	0.0185	17	-0.0154	0.0625
	5	*a*	0.0017	0.0004	16	0.0008	0.0026
		b	0.0120	0.0135	16	-0.0167	0.0407
	6	*a*	0.0018	0.0005	17	0.0007	0.0029
		b	0.0556	0.0185	17	0.0166	0.0945
	7	*a*	0.0011	0.0004	16	0.0003	0.0020
		b	0.0437	0.0232	16	-0.0055	0.0930
	8	*a*	0.0034	0.0009	14	0.0016	0.0052
		b	0.0884	0.0235	14	0.0379	0.1389

[1] Estimates of the parameters for intercept (*a*) and slope (*b*) were significant ($p < 0.0001$).
[2] Standard error for the estimated parameters.
[3] Degrees of freedom.
[4] The 95% confidence interval for the estimated parameters.

Table 4-A2. Results of the regression analyses of the outcrossing experiment at Westwold B.C. with the data from each block.

Experiment site	Block	Parameter	Estimate[1]	Standard error[2]	Df[3]	95% confidence interval[4] Lower	Upper
Westwold	1	*a*	0.0156	0.0014	31	0.0128	0.0184
		b	0.0569	0.0035	31	0.0496	0.0641
	2	*a*	0.0138	0.0013	31	0.0110	0.0165
		b	0.0469	0.0035	31	0.0398	0.0541
	3	*a*	0.0175	0.0017	31	0.0139	0.0210
		b	0.0706	0.0052	31	0.0600	0.0812
	4	*a*	0.0257	0.0029	31	0.0197	0.0316
		b	0.0765	0.0047	31	0.0197	0.0860

[1] Estimates of the parameters for intercept (*a*) and slope (*b*) were significant ($p < 0.0001$).
[2] Standard error for the estimated parameters.
[3] Degrees of freedom.
[4] The 95% confidence interval for the estimated parameters.

Table 4-A3. Results of the regression analyses of the outcrossing experiment at Lethbridge, AB with the data from each block.

Experiment site	Block	Parameter[1]	Estimate[2]	Standard error	df[3]	95% confidence interval Lower	Upper
Lethbridge	1	*a*	-	-	-	-	-
		b	-	-	-	-	-
	2	*a*	0.00002	0.00006	34	-0.00011	0.00015
		b	0.00442	0.09261	34	-0.18380	0.19260
	3	*a*	-	-	-	-	-
		b	-	-	-	-	-
	4	*a*	0.00002	0.00004	33	-0.00006	0.00010
		b	-0.03511	0.04600	33	-0.12870	0.05847
	5	*a*	0.15330	7.27830	34	-14.63800	14.94460
		b	15.49220	189.90000	34	-370.43000	401.41000
	6	*a*	0.00033	0.00036	33	-0.00040	0.00107
		b	0.07397	0.04079	33	-0.00902	0.15700
	7	*a*	0.01403	0.00274	34	0.00847	0.01959
		b	0.34380	0.04812	34	0.24600	0.44160
	8	*a*	-	-	-	-	-
		b	-	-	-	-	-

[1] Estimates of the parameters for intercept (*a*) and slope (*b*) were significant ($p < 0.0001$).
[2] The regression analysis did not converge for blocks 1, 3 and 8 because most of the observed frequencies were zero values.
[3] Degrees of freedom

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Chapter 5: Potential for seed-mediated gene flow in agroecosystems from transgenic safflower (*Carthamus tinctorius* L.) intended for plant molecular farming

INTRODUCTION

The oilseed crop safflower (*Carthamus tinctorius* L.) has been transformed with a construct with the intention of field scale production of high-value proteins for use as pharmaceuticals and industrial enzymes (plant molecular farming, PMF). Mitigation of gene flow will be a requirement, particularly for PMF where the products may be substantially different from commodity products, and may need to be stringently segregated from conventional food and feed systems. While considerable interest has been directed to transgene flow via pollen (Poppy and Wilkinson, 2005), the importance of gene flow via seed is frequently overlooked. Crop seed is a propagable material as well as a major commodity, which may be distributed locally and internationally (Saji et al., 2005) with consequence to international trade. Volunteer populations of transgenic canola (oilseed rape, *Brassica napus* L.) have been found, initiated by seed spill during transport (Saji et al., 2005; Yoshimura et al., 2006) and weed and crop seed dispersal by farm machinery in and among fields is well documented (Barroso et al., 2006; Blanco-Moreno et al., 2004; Shirtliffe and Entz, 2005). Seeds may germinate, producing volunteer plants and the seeds from these populations may serve as secondary sources of gene flow (Yoshimura et al., 2006). In addition, seed harvested from volunteer transgenic crop plants in following years may be admixed (commingled) with food or feed crops.

The potential for seed mediated-gene flow of safflower under Western Canadian conditions is not known.

Safflower is a member of the Asteraceae in the thistle tribe (Cardueae) originally domesticated in the Mediterranean. Historically it has been grown for dyes extracted from florets and the seeds used for food / cooking oil. The safflower seed (achene) is similar to sunflower (*Helianthus annuus* L.) in appearance, histology and oil body transmembrane protein make-up (oleosins), and is approximately the size of a large barley seed (Lacey et al., 1998; Mundel et al., 2004; Vaughan, 1970). Cultivated varieties range in seed oil content from 24 to 40% (Vaughan, 1970). Efforts to breed safflower lines with more rapid maturity to reduce risk of crop failure on the Canadian prairies were successful but seed oil content is below requirements for food and industrial oil. In Canada, safflower line 'Saffire' has white seeds that are attractive for bird seed markets and it is grown on limited acreage (Mundel et al., 2004).

The persistence and viability of crop seed in the seed bank is known to vary with the number of viable seeds in the soil and/or the microsite for each seed (i.e. depth of burial and soil moisture) (Boyd and Van Acker, 2004; Cummings and Alexander, 2002). Generally, annual crop seeds do not persist for long periods of time in soil (Cavers and Benoit, 1989). The persistence of canola has been shown to ranging from 4 to 5 years (Simard et al., 2002), whereas wheat persistence has been shown to range from 2 to 5 years (Anderson and Soper, 2003; Harker et al., 2005; Kremer, 1993). However, safflower persistence in the western Canadian agroecosystem has not been studied.

Sources of crop seed bank inputs include the initial seeding of the crop, and more importantly loss of seed prior to and during harvest. Shatter (seed release) prior to harvest

is a feral trait that is usually selected against during crop domestication (Harlan, 1992). Cultivated safflower is highly shatter resistant compared to its wild relatives (Berville et al., 2005; McPherson et al., 2004; Mundel et al., 2004). However, seed head loss due to pathogen infections, insects, and bird and mammal predation still occurs.

Harvest losses of crops are highly variable and can be sizable. Safflower is generally harvested without prior swathing and even with optimal combine adjustments and operation, harvest losses occur at the header and in residue and chaff. Harvest losses for safflower have been estimated at 3 to 4% for yields of 2200 to 3400 kg ha^{-1} (approximately 1.4 to 2.8 seeds m^{-2}) in California when the combine adjustments are optimal and safflower is at *ca.* 9 % moisture; (Knowles and Miller, 1965). Alberta safflower growers' harvesting methods include harvesting safflower at 12 to 15 % moisture to reduce losses and ensure a timely harvest. Grain dryers are used to decrease the seed moisture to 9.5% while maintaining seed quality (Mundel et al., 2004). Information is lacking on pre-harvest and harvest losses of safflower seed in Canada.

Predation can result in large seed losses from the seed bank for crop seeds lost during harvest. Seeds are a food source for a range of species, including mammals, birds and invertebrates. While it is understood that seed predation is a significant factor in seed bank depositions and loss, estimates of loss due to predation are limited to a few plant species, predominantly trees and weeds (Cummings and Alexander, 2002; Davis et al., 2005; Louda, 1989) and have not been investigated for safflower.

Seed dormancy can influence the persistence of viable seed in soil. A key crop domestication step is often the loss of seed dormancy (innate and induced) that facilitates synchronous germination of crop seed following planting (Harlan, 1992). However,

innate and induced dormancy are adaptive characteristics that promote the survival of many weedy species (Baker, 1974). Safflower is one of the oldest crops and has been selected for reduced dormancy (Berville et al., 2005). Some early breeding efforts to adapt safflower to North America involved increasing innate (primary) dormancy to prevent seeds from germinating in the heads and, sprouting resistance, prior to harvest. Seed dormancy of wild safflower biotypes is controlled by several loci and hybrids among them and cultivated safflower have increased levels of innate dormancy (Kotecha and Zimmerman, 1978; Zimmerman, 1972). Dajue and Muendel (1996) summarized germination tests conducted on several cultivated varieties of safflower after harvest. They found that 60% or more of the seed would germinate 121 to 175 hours after harvest. The degree, if any, of innate or induced seed dormancy for the safflower variety (Centennial) under consideration for PMF has not been reported.

Seed placement in the seed bank influences the environment the seed encounters (Davis et al., 2005), and placement of seeds shed during and after harvest varies with tillage practices. Depth of placement influences light, temperature and moisture, all of which may influence germination (Cavers and Benoit, 1989). When planted as a crop, safflower germination requires more moisture than cereals in Alberta agroecosystems and it does not germinate well on the soil surface (Mundel et al., 1995). Safflower germination is poor when soil temperatures are below 5 °C, but the incidence of damping-off increases when soil temperatures are above 10 °C, especially when soil moisture is high (Mundel et al., 1995). Safflower seeds remaining in fields after harvest may succumb to inappropriate germination; germination under conditions where plant success is limited. Safflower is not frost resistant even after considerable efforts to breed

types for winter cropping (Mundel et al., 2004; Zimmerman, 1972) and if seeds germinate in the fall, they will not survive the Canadian winter.

Safflower seed on the soil surface may be distributed in fall or spring by tillage or by seeding operations. Without fall tillage, most seeds remain on the soil surface and may be subject to poor soil contact and desiccation. Optimum spring seeding depth for safflower in Alberta is 2 to 3.5 cm. Although safflower has a large seed, seedlings planted deeper are more likely to be infected by *Pythium* spp., a pathogen that causes seedling damping-off (Mundel et al., 1995). Deep seeding may also extinguish energy reserves before emergence leading to reductions in plant density and seedling survival (Mundel et al., 2004). Weed seed banks have been shown to be reduced by microorganisms in agroecosystems (Kremer, 1993) and their seed energy reserves may also be exhausted over time, although it is known that some weed seeds can remain viable for decades (Conn et al., 2006). The persistence of safflower seed lost at harvest in fields has not been quantified.

Smith (1996) suggests that safflower volunteers do not become a pest in subsequent crops and if present, occur during the first year following production. In the United States, safflower has been documented to have volunteered after cultivation in Arizona, California, Colorado, Idaho, Iowa, Illinois, Kansas, Massachusetts, Montana, Nebraska, New Mexico, North Dakota, Ohio Oregon, Utah and Washington (Berville et al., 2005; Keil, 2006; McPherson et al., 2004). In Canada safflower has been reported as escaped cultivation in Alberta and British Columbia. In most cases it is not known how long these populations of safflower persist outside of the agroecosystem. Safflower is a poor competitor with weeds due to slow growth of the rosette stage early in the season

(Blackshaw, 1993). I hypothesized volunteer survival and fecundity would be highly reduced in competitive follow crops such as barley or wheat. In addition, safflower is not tolerant of many herbicides, and those commonly used on following crops, especially in cereal crops, are likely to limit safflower survival and fecundity (Blackshaw et al., 1990a; Blackshaw et al., 1990b). Safflower volunteers have not been found in the three large scale weed surveys of cultivated land on the Canadian Prairie Provinces (Alberta, Saskatchewan, and Manitoba) conducted to date (Leeson et al., 2005). These surveys have documented other crop volunteers to occur at high frequency. For example, canola (rapeseed, *Brassica napus* L.), wheat (*Triticum aestivum* L.), flax (*Linum usitatissimum* L.) and barley (*Hordeum vulgare* L.) volunteers have been ranked among the top 50 most abundant weeds in arable fields from the 1970s to 2000s. However, safflower is grown by a small number of producers on the Canadian prairies and these fields may not have been included in the field surveys. Thus these surveys are an inappropriate method to estimate volunteerism of a minor crop. Safflower has been grown as a minor crop in Canada since 1943 (Blackshaw et al., 1990b). Experienced growers do not express concern with volunteers, and it is absent in descriptions of the flora (Berville et al., 2005; McPherson et al., 2004) suggesting this crop does not persist or escape the Canadian prairie agroecosystems extensively. Safflower volunteerism and success (survivorship and fecundity) has not been quantified under Alberta conditions.

To estimate seed-mediated gene flow as part of a biosafety assessment of safflower for PMF production, I evaluated the density of seed deposited on fields following harvest of the commercial early maturing safflower cv. 'Saffire'. In addition, I conducted experiments to compare transgenic and non-transgenic safflower cv. 'Centennial' seed

longevity in the seed bank. Commercial fields previously cropped to safflower ('Saffire') were surveyed, collecting measurements of safflower volunteer emergence and stage of development to provide estimations of volunteer seedling survivorship and fecundity under production conditions.

Although safflower was among the first crop species to be domesticated, aspects of its biology are not well understood. Recommendations by Canadian regulators for PMF have included the use of crop species not cultivated for food or feed (CFIA, 2007). Safflower may be an appropriate platform for PMF because it is a domesticated crop grown on a low acreage in Canada, and no cross-compatible wild relatives exist in the area (McPherson et al., 2004). However, potential for seed-mediated gene flow of safflower in the Canadian prairie agroecosystem has not been determined because the number of seeds lost at harvest, and their persistence and potential to produce volunteers has not been quantified. The present study can be used to determine the length of time that monitoring (survey and locate) and control of volunteers will be required for land used for PMF safflower in post-harvest years. In addition, it will evaluate contamination of follow commodity crops, and assist in the development of best management practices to mitigate such an occurrence. Co-existence of commodity agriculture and plant molecular farming will require confinement and segregation of both commodity and PMF safflower types in the agroecosystems and during grain handling after harvest.

RESULTS

Safflower harvest losses from commercial fields

To estimate the seed loss at harvest in commercial safflower fields I collected seed immediately following harvest in five fields (designated D, E, F, I and J) at two sites (Warner and Wrentham) in southern Alberta in the fall of 2005 (Tab. 1). The density of safflower seed recovered from the three fields in Warner ranged from 231 and 614 seeds m^{-2}, whereas, at Wrentham it was 315 to 1069 seeds m^{-2}. However, the density of viable seed was lower, ranging from 125 to 518 seeds m^{-2} at Warner and 81 to 515 seeds m^{-2} at Wrentham (Tab. 1) which is approximately 1 to 5 times the recommended seeding rates for safflower (*ca.* 100 to 150 seeds m^{-2}) (Mundel et al., 2004). The percentage of viable seeds to total seeds lost ranged from 25.7 to 84.3 % with the lowest in field J and the highest in field D (Tab. 1). Thus, safflower harvest losses were highly variable in each field, and significant differences occurred among fields at each farm site and among the farm sites.

Seed persistence and viability

Seed was sown into rodent-proof nylon mesh envelops and buried at three depths and two locations in Alberta. Seed was withdrawn at intervals over subsequent growing seasons and viability tested. A regression analysis of the number of viable seeds over time was conducted using a non-linear exponential decay function for all experimental effects. Parameter estimates from each regression for the rate of decline (*b*) were subject to ANOVA to determine significant differences among experimental effects. Analysis of variance for the rate of seed viability decline showed consistency among years

(p=0.9820) and a significant interaction of site and depth among years (p=0.0469). Thus, subsequent analyses were conducted on separate years.

Burial depth significantly influenced safflower seed viability. Seeds on the surface declined in viability at a slower rate than those buried at 2 and 15 cm (Tab. 5-2 and Fig. 5-2 and 5-3). The rate of decline of seed viability between the burial treatments was significantly different in 2004 ($p = 0.0013$) but not 2002 ($p = 0.1846$). The influence of burial depth on the rate of safflower seed viability decline varied with environment. Seed burial depths were significant at all sites and years of trial establishment ($0.005 < p < 0.0001$) except in 2002 at Warner ($p = 0.0596$). When burial depth was significant, surface seeds persisted longer than buried seeds ($0.0398 < p < 0.0001$). Seed viability decline rate was more rapid for buried seeds in Warner than at Ellerslie ($p=0.0184$) in 2004 but not 2002 ($p = 0.1686$ and Fig. 5-1). The response of seed rate of decline by burial treatment differed among the sites (interaction $p = 0.0063$). The rate of decline was similar for seeds at the surface and 15 cm depth at both sites, but was more rapid for seeds at Warner than Ellerslie at the 2 cm depth ($p = 0.005$). The more rapid decline of buried safflower seeds is possibly a result of increased contact with soil and decreased desiccation. Thus, seeds are more likely to leave the viable seed bank by germination, increased metabolic activity and exhaustion of energy reserves or microbial infection.

Viability of safflower seeds lost at harvest would be diminished over the Canadian prairie winter as indicated by the estimates for the time for 50% reduction of seed viability (seed extinction, EX_{50}) and estimates of percent viable seeds in spring (May 1, Tab. 5-2). The EX_{50} values for seeds at the surface ranged from 87.8 to 119.4 days after insertion (mid-winter to early spring) and those for buried seeds ranged from 24.9 to 43.6

days after insertion (early to mid-winter). Thus, safflower seeds lost 50% of their viability at all depths, sites and years, prior to the time a spring follow crop would be planted (Tab. 2). Safflower seed viability in early spring after trial establishment (May 1) was below 50%. The percentage of viable seed on the surface in spring was 38 to 43 % in 2002 and 26 to 30 % in 2004, whereas, the percentage of viable buried seeds in spring was 2 to 10 % in 2002 and 0.9 to 5.2 % in 2004 (Tab. 2).

The viability of safflower seeds lost at harvest and surviving the winter would be further diminished in the first follow year indicated by estimated time for seed viability to be reduced by 99% (EX_{99}) and percentage of seed survival in the first fall after planting (September 1) (Tab. 2). Generally, safflower seeds had a rapid degradation of viability, reaching EX_{99} when on the surface between 629 days (second mid-summer after burial) and 810 days (second winter after burial) after fall insertion (Tab. 2). When buried, this was reduced to between 172 days (prior to spring after planting) and 298 days (late summer to fall after burial). At the end of one growing season (September 1), only 21.3 to 10.7 % of seeds at the surface were viable and 1.5 to 0 % of the buried seeds remained viable at all sites in all years (Tab. 2).

Rate of viability decline for transgenic and non-transgenic safflower was different on one occasion at the Ellerslie trial established in 2004 ($p = 0.0064$). Non-transgenic seeds declined more rapidly than transgenic seeds at 15 cm ($p = 0.0032$) and marginally so at 2 cm ($p = 0.05$). This was not due to greater seed viability of the transgenic seeds at the time the trials were established. Both genotypes responded the same on the soil surface (Tab. 5-2 and Figs. 5-2).

Survey of commercial safflower fields

Surveys of commercial fields in the two years following safflower production were conducted to quantify safflower volunteer emergence, survival and fecundity. Safflower volunteer plant density in the early spring of 2005 ranged from 0.04 to 1.7 plants m^{-2} at Warner and 0.029 and 0.07 plants m^{-2} at Wrentham (Tab. 3). In 2006, early spring safflower volunteer densities at Warner ranged from 2.3 to 10.9 plants m^{-2} and at Wrentham from 2.9 to 10.3 plants m^{-2} (Tab. 4). Safflower volunteer densities in fields surveyed in 2006 accounted for 1 to 9% of the viable seed lost at harvest and quantified in the fall of 2005 (Tab. 4). Safflower volunteer densities were significantly reduced by herbicide applications and follow crop seeding operations (Tab. 3 and 4). On some occasions, safflower volunteer plants were documented in surveys conducted after dates where no safflower was found, suggesting that safflower plants continued to emerge throughout the growing season. Fallow herbicide applications prevented safflower plant volunteer survival beyond the bud stage (BBCH 53) and consistently reduced plant densities to zero at the end of the growing season (Tab. 3 and 4). In several cases during surveys of follow cereal crops prior to harvest, no safflower volunteers were detected but at Warner safflower volunteers survived and set seed in a wheat (field A) and two barley fields (fields E and F) in 2005 and 2006, respectively (BBCH scale 99 and 87, respectively) (Tabs. 3 and 4). These plants produced a mean of between 2.2 to 7.9 seeds $plant^{-1}$, much less than cropped safflower in neighboring fields which produced a mean of between 160 and 236 seeds $plant^{-1}$ (Tab. 5).

The cultivated variety of safflower grown in these fields, 'Saffire', requires between 115 and 147 days to reach reproductive maturity, but volunteers matured more quickly

(Tab. 3 and 4). For example, no safflower plants were found in the Warner barley fields E and F in 2006 when surveyed on May 4, but plants emerging after this date set seed by August 1 which was only 69 days later (Tab. 4). Cropped safflower at the same farm site (Warner) matured after 1050 cumulative growing degree days (GDD), whereas the plants that matured to seed set in fields E and F did so within 820 GDD (Tab. 4). The recommended seeding dates for 'Saffire' in these areas is between the first week in April and May as later planting dates result in reduced seed and oil yields. Later seeded 'Saffire' requires fewer days for maturity, but the number of heat units required for maturity without a yield penalty remains the same (Mundel et al., 1992). Thus, safflower exhibits a strong phenotypic response to competition from a follow crop by quicker maturity than cropped safflower (Tab. 5), and set viable seeds (below).

Comparison of volunteer safflower to crop safflower plants

Safflower volunteer plants, when present at the time of the follow crop harvest, were hand harvested along with safflower in nearby fields at the same farm site. Characteristics of safflower volunteer versus crop plants were generally highly significantly ($p < 0.001$) Exceptions were non-significant plant height in 2006 ($p = 0.0672$), and in 2005 harvest index was slightly less significant ($p=0.0013$) (Tab. 5). In 2006 two barley fields separated by less than two kilometers had safflower volunteers at the time of harvest but the characteristics measured did not differ among these fields. Thus, they were compared as a group to the crop plants sampled at this farm in the same year (Tab. 5).

Comparison of volunteer safflower in cereal follow crops with safflower crop plants grown at the same farm site confirmed that safflower is a poor competitor with both wheat and barley. Volunteer safflower plants relative to crop safflower plants were

reduced in height, number of heads plant^{-1}, total number of seeds head^{-1} and plant^{-1}, number of viable seeds plant^{-1}, seed kernel weight, plant biomass and harvest index (Tab. 5). Thus, volunteer safflower surviving in follow cereal crops relative to its cropped counterpart had a significant reduction in fecundity. Volunteers set 95% less seed plant^{-1} relative to cropped safflower and averaged 50% germination under ideal conditions prior to exposure to winter.

DISCUSSION

Under normal production practices, volunteer safflower populations did not persist beyond two years in Alberta. Safflower seed losses at the time of harvest ranged from 230 to 1070 seeds m^{-2}, well in excess of the recommended seeding rate of 100 to 150 seeds m^{-2}. The viability of these seeds ranged from 80 to 520 seeds m^{-2} (26 to 85%). Seed burial experiments indicated that seed viability declined sharply over the first winter with EX_{50} values between 24.9 and 119.4 days after insertion, corresponding to fall through early spring (November 27 to April 3). Seed viability continued to decline throughout the growing season with estimates of viable seed in early fall ranging from 0.00% to 21.3% (Tab. 2). Volunteer densities from the commercial fields surveyed were low in the first spring following the safflower crop, relative to seed losses, ranging from 0.029 to 10.91 plants m^{-2} (Tab. 3 and 4). Cultural practices decrease the survival and fecundity of volunteers. In seven of the ten fields surveyed, volunteers did not survive to set seed. When volunteers did mature prior to follow crop harvest, they were at low densities (0.05 to 0.33 plants m^{-2}) and produced few viable seeds (1.2 to 4.1 seeds plant^{-1}). Multiplication of these values suggests limited seed bank replenishment, 0.06 to 1.353 seeds m^{-2}, in any field. No volunteers were identified in the second follow growing season. Thus, the seed

bank was depleted and volunteers did not germinate or they were present in such low densities that they were not detected.

Harvest losses of viable safflower were highly variable between fields and were equal to or above recommended seeding rates for this crop. Safflower in these fields is marketed for birdseed and low bushel weights are undesirable. Thus, combine settings are adjusted to remove under-filled and immature seeds and impurities, and losses would be expected. Harvest losses for canola have been documented and ranged from 1,500 to 7,100 seeds m^{-2} with an overall average of 3,000 viable seeds m^{-2} (Gulden et al., 2003a). Wheat harvest losses can range from 35 to 800 seeds m^{-2} with an approximate average of 300 seeds m^{-2} (Anderson and Soper, 2003; Clarke, 1985; De Corby et al., 2007). Although safflower harvest losses were high, they were much lower than canola and within the range of wheat.

There was little indication that transgenic safflower persisted longer than its non-transgenic counterpart. Safflower seed viability in the burial trials was reduced more than 50% during winter by inappropriate germination, disease, and other factors. Safflower seed germinability was zero at the end of the second growing season for seeds recovered from the burial trials in 2002, whereas viable seeds did not germinate beyond the second spring after burial from the 2004 burial trials. This suggests the seeds were alive but lacked the energy to germinate. In addition, no evidence for extensive dormancy was found for safflower in these trials. By comparison, 44% of high dormancy canola seed lost at harvest remained viable after one winter and 0.2% were viable after three winters (Gulden et al., 2003b). Seed banks of wheat established by losses at harvest can remain viable for 12 to 18 months (De Corby et al., 2007; Harker et al., 2005). Thus, transgenic

and non-transgenic safflower seeds behave similarly to other domesticated crops grown on the Canadian prairies.

Burial of safflower seeds significantly increased their loss from the seed bank relative to seeds at the surface most likely by increasing soil contact and moisture content and reducing desiccation. Thus, the buried seed bank is more rapidly diminished by germination, and microbial infections, and through a decrease or exhaustion of energy reserves by an increased seed metabolism. Safflower growers in Alberta practice zero tillage management. Therefore, few of the safflower seeds lost at harvest would be buried. Thus, the longevity of these seeds would be similar to those in the surface treatments of the burial studies and would follow a similar extinction curve. The EX_{50} for the majority of these seeds occurred mid-winter to early spring of the following year (Tab. 2; *ca.* January to April) thus less than half of the seeds are viable in spring. In follow years, the seeding operation in a zero tillage regime would bury a proportion of seeds (depending on the seed bed utilization) (De Corby et al., 2007). However, if fall tillage were adopted after cultivation of safflower, a larger proportion of the seeds would be buried. Based on the burial studies the EX_{50} of these seeds would be early-winter (November to January). Prior to planting in spring, the proportion of viable seed would be considerably lower than for untilled fields.

The burial studies and field surveys provide different estimates of safflower seed longevity in the agroecosystem. The disparity between the artificial burial studies, and the field surveys suggest that artificial seed banks overestimated seed viability relative to natural seed banks as has been shown for some weed species (Leon and Owen, 2004). A

meta-analysis including 599 species conducted by Thompson et al. (2003) has shown that burial and extraction (artificial burial) studies over estimate longevity of seeds in soil.

Safflower volunteers in spring were at low densities, similar to other crop volunteers in the Canadian prairies. Abundance of other crop volunteer quantified in Canadian prairie fields from the 1970s to 2000s, when they occurred, ranged in densities from 3.4 to 4.0 plants m^{-2} for barley, 3.9 to 5.9 plants m^{-2} for wheat, 4.5 to 28 plants m^{-2} for canola; and 8.7 to 34.3 plants m^{-2} for flax (Leeson et al., 2005). Safflower volunteers in the fields surveyed here, when they did occur, ranged from 0.03 to 17 plants m^{-2}. Safflower volunteers were not detected in the second follow year after its production, in the field surveys of commercial fields. In PMF safflower field trials conducted over 10 years where volunteers are controlled in the first year prior to flowering to meet government regulatory conditions no volunteers have been reported in the second year.

Surveying commercial safflower fields allowed volunteer data to be collected over a range of conditions. Most safflower volunteer plants in follow cereal crops did not survive and set seed, presumably due to plant competition and herbicide effectiveness. However, some safflower volunteers in wheat and barley were reduced in several characteristics relative to cropped safflower and produced some viable seeds. The influences and response of volunteer safflower in follow crops to competition and herbicides and the amount of admixture (commingling) of transgenic safflower during follow crop harvest have been addressed specifically in another study (Chapter 6).

Viability of safflower volunteers' seed set in cereal crops in 2005 and 2006 was low, relative to cropped safflower, *ca.* 50% and ranged from 1.2 to 4.1 viable seeds $plant^{-1}$. In addition, the mean seed weight of volunteer safflower was highly reduced relative to

cropped safflower and perhaps these smaller seeds would contain less energy and not persist as long as larger ones. Thus, these safflower volunteers did produce a small recharge of the seed bank but few seeds would be expected to survive and produce volunteers in the following growing season. Seed bank recharge has been shown to be a driving factor for volunteer persistence in other crops. Volunteer canola has been shown to persist for up to five years after harvest loss on the Canadian prairies (Simard et al., 2002), but when volunteer seed production is suppressed canola seed banks can be rapidly depleted in 3 years (Harker et al., 2006). Wheat volunteers have been observed up to two years after harvest (Anderson and Soper, 2003; Harker et al., 2005). However, if wheat volunteers are allowed to set seed and replenish the seed bank, populations have been documented to persist for as long as five years (Beckie, 2001). Volunteer control will be critical to reduce seed mediated gene flow from PMF safflower.

These three diverse studies of safflower harvest loss, seed persistence and volunteerism demonstrate that safflower does not persist beyond two growing seasons after production. It should be noted that experienced growers managing the surveyed commercial safflower fields were not specifically concerned with controlling safflower volunteers and field data reflects safflower biology under normal zero tillage management conditions. Management practices can be modified to limit safflower seed-mediated gene flow in the agroecosystems following production. During harvest the number of viable seeds lost to the soil surface should be minimized. Tillage following harvest would increase seed burial, minimize seed persistence and likely reduce predation of seeds substantially (Westerman et al., 2006). Chemical fallow the year following PMF safflower production would control safflower volunteers, prevent the production of

pollen and seed bank recharge and facilitate monitoring. Seed mediated gene flow from transgenic safflower volunteers is limited and modified management practices during and after production can be employed to mitigate its occurrence.

MATERIALS AND METHODS

Source of plant materials

To assess the environmental influences on safflower seed persistence in Alberta agroecosystems and the potential for safflower volunteers in follow crops, I conducted trials and field surveys of commercial fields at Ellerslie and Warner, Alberta from 2002 to 2006. Seeds for both the transgenic and non-transgenic safflower were provided by SemBioSys Genetics Inc. (Calgary, Alberta, Canada). Seed lots were tested for cross contamination by greenhouse screening with glufosinate and were true to type.

Transgenic and non-transgenic safflower seeds used for the burial trials in 2002 were produced in Pirque, Chile and Enderby, BC, Canada, respectively. Seeds for both genotypes were increased under identical greenhouse conditions and used for the burial trials established in the fall of 2004. All seed lots were tested for the ability to germinate and for viability using a tetrazolium test (see below).

Environmental biosafety compliance

For all of the transgenic trials conducted, we stringently adhered to protocols for containment outlined by the Canadian Food Inspection Agency (CFIA, 2006). These protocols included appropriate isolation distances of transgenic trials from commodity safflower crops, isolation of trials by 50 m to any commodity crops, triple containment of seed to and from the sites, and careful cleaning and inspection of all equipment used at

the sites. They also required frequent monitoring and control of safflower volunteers during the trials and post-harvest trial sites, and a 10 m safflower free perimeter for two subsequent years.

Safflower harvest losses from commercial fields

In the fall of 2005 after producers harvested their commercial safflower, the density and viability of seed losses at harvest were measured by taking samples along a diagonal transect across each field. Every 50 to 120 m, seed and residual plant material were removed from a 1 m^2 area using an industrial vacuum cleaner. The samples were later cleaned by hand and soil sieves to retrieve safflower seeds. Seeds were tested for viability as outlined below.

Safflower seed bank persistence experimental design and environments

Experiments were initiated in the fall of December 2, 2002, and November 3, 2004, at the Ellerslie Research Farm, Edmonton, Alberta, Canada (N53° 25″ W113° 33″) and a farm near the town of Warner, Alberta, Canada (N49° 19″ W111° 57″). Each trial was planted as a split plot in a randomized complete block design with depth as the main plot and genotype (transgenic and non-transgenic) as the subplot. Two hundred seeds of transgenic and conventional safflower of the same variety, 'Centennial', were sown into separate 10 x 10 cm nylon bags. Each plot had ten pairs of transgenic and non-transgenic seed packets randomly placed with 5 cm spacing. Each of the three replicates consisted of three randomized 1 m^2 plots at 0, 2 and 15 cm depths. Initial seed viability for each trial is listed in Table 5-2.

Extraction and processing of seeds from safflower seed bank persistence studies

Throughout the subsequent growing seasons paired samples, transgenic and non-transgenic, were randomly extracted from each plot in the early spring, twice in mid-summer and again in the fall, at approximately six week intervals. Seeds were stored at 4 °C for two to five days prior to processing. Excess soil was washed from the bags and seeds removed. The number of seedlings, soft or degraded seeds, seed coats (pericarp) without embryo and un-germinated intact seeds were recorded from each packet. Any un-germinated seeds were then tested for viability (below).

Seed viability testing

Seeds were placed in acrylic germination boxes (24 x 16 x 3.8 cm) (Hoffman Manufacturing, Inc.) between 15 x 23 cm non-toxic white filter paper equivalent to Whatman #1 (Hoffman Manufacturing, Inc.). To reduce fungal growth, 14 mL of a 0.2 % solution of the seed treatment Helix XTra ® (thiamethoxam + difenoconazole + metalaxyl-M + fludioxonil) was added to each germination box. Previous experiments with this solution showed that it did not inhibit germination of safflower seeds (data not shown). The germination trays were stored in the dark at ambient temperature for four to five days. Seeds with emerged radicals were considered germinated. The seeds that did not germinate were cut along the pericarp (seed coat) suture line and placed embryo side down in 0.015 % tetrazolium chloride and incubated for 2 hours at 30 °C (Porter et al., 1947). Seeds with a positive tetrazolium test were considered viable and those with a negative result as dead.

Field surveys of commercial fields

Field surveys were conducted in 2005 and 2006 in commercial fields cropped to safflower cv. 'Saffire' the previous season. Each year, five fields were surveyed in southern Alberta close to the towns of Warner and Wrentham. In the year following production, safflower volunteer densities and developmental stages were estimated in each field over the course of the growing season. Safflower volunteer density was determined in four 20 m x 1 m areas in each field (80 m^2 total). If five or fewer plants were found in any of these surveyed areas then another two v-shaped areas of 100 m x 1m were surveyed (200 m^2). If during this second surveying interval, safflower plants were not found, an additional 200 m^2 were surveyed. Mean safflower stage was described for each field using the sunflower BBCH-scale (Meier, 2001).

Comparison of volunteer safflower to crop safflower plants

Density and growth stage for safflower volunteers were documented. Prior to harvest individual plants were harvested and measurements taken of plant height, number of branches, heads and seeds per plant, biomass, thousand kernel weight and seed viability. Harvest index was calculated as the ratio of total seed weight and biomass per plant, and mean number of seeds per head was calculated as a ratio of total number of seeds and heads per plant. Seed viability was assessed after storage for 24 weeks or longer at ambient temperature to ensure the breaking of potential primary dormancy (*ca.* 2 to 24 weeks) that has been documented for hybrids of wild and cultivated safflower (Kotecha and Zimmerman, 1978).

Statistical analysis

Safflower harvest losses from commercial fields

The density and viability of safflower seeds recovered from the soil surface in commercial fields after harvest were subject to mixed model ANOVA (SAS, 2007) with fields nested in farm site. Two separate analyses were conducted with fields as random and fixed effects in the model, in order to extrapolate to future years and fields and to make comparisons, respectively.

Safflower seed bank persistence

The number of viable safflower seeds remaining in the seed bank was calculated as the sum of the germinated and tetrazolium positive seeds. Initial analyses of variance were confounded by heterogeneity of variances and a large number of samples without any viable seeds (zero values). The frequency of viable seeds (y) at each extraction time days after planting (d) were subject to a regression analysis using a nonlinear regression mixed model (PROC NLMIXED) with Statistical Analysis Software (SAS, 2007). The binomial distribution (~ binomial (n, y)) was employed to approximate the dependent variable where n is the total number of seeds buried and y is the number of viable seeds recovered at each sampling date. Various equations from the literature were assessed for goodness of fit in the nonlinear regression analysis, but the simple exponential decay given in equation 1 best fit the data as determined by model convergence.

$$y = ae^{-bd} \qquad (1)$$

where y is the frequency of viable seeds, a is the intercept, b is a shape parameter describing the rate of decline and d is the number of days after planting.

Regression analysis was conducted on the individual factors of year, site, replicate (block), burial depth and genotype. The parameter estimates for the rate of decline (b) for each regression were analyzed with ANOVA, using a split plot mixed model (SAS, 2007) with depth as main plot and genotype as a subplot. Years were considered random in the analysis, whereas sites were considered a fixed effect because they were specifically chosen to evaluate the influence of these two distinctly different environments. When factors were not significant in the ANOVA, they were grouped and subject to nonlinear regression. Using a natural logarithm transformation of equation 1, I estimated the time to extinction of viability for 50% and 99% of the seed (EX_{50} and EX_{99}; equation 2 and 3, respectively).

$$EX_{50} = \frac{[\ln(0.5 * a) - (\ln a)]}{-b} \qquad (2)$$

$$EX_{99} = \frac{[\ln(0.01 * a) - (\ln a)]}{-b} \qquad (3)$$

where a is the intercept and b is a shape parameter describing the rate of decline.

To evaluate the influence of winter and summer on the buried safflower seeds, the frequency of viable seeds in the first spring (May 1) and fall (September 1) after planting was estimated. These estimates were derived by substituting the number of days after planting corresponding to May 1 and September 1 and the regression estimates for intercept and rate of decline into equation 1.

Field surveys of commercial fields

Density of safflower volunteers in follow crops were subject to ANOVA for each year separately (SAS, 2007). The model included farm site with fields nested within their respective farm site. Each sampling date was analyzed separately to facilitate

comparisons of fields over time. Means were separated using least significant difference (LSD) with the pdiff option in SAS.

Comparison of volunteer safflower to crop safflower plants

Data collected from safflower volunteer and safflower crop plants (type) at the time of follow crop harvest were subject to a mixed model ANOVA (SAS, 2007). The model included crop and volunteers as fixed terms and incorporated the random effects of years and fields nested in years. The tests were one tailed with the null hypothesis that safflower crop plant characteristics were not consistently larger than volunteer safflower. In several cases fields within years were significant, thus subsequent analysis were conducted on separate years. To determine if there was significant heterogeneity among the fields, a log-likelihood ratio test or G-test using the chi-square distribution was conducted using the -2 log-likelihood score provided by SAS for analysis conducted with and without a repeated statement with field as the subject and type (crop and volunteer safflower) as the group to account for heterogeneity of variances (SAS, 2007; Zar, 1999). In all cases, the model with heterogeneity of variances was a significant improvement except for harvest index, which was analyzed with the assumption of homogeneity of variances.

Table 5-1. Safflower seed recovered from fields at Warner and Wrentham after harvest in the fall of 2005.[1]

Site	Field	Total Seed m^{-2} (SE)		Viable Seed m^{-2} (SE)		Seed loss[2] %
Warner	D	614 (210)	a,b	518 (112)	a	84
	E	231 (210)	a	125 (112)	b	54
	F	328 (210)	a	134 (112)	b	41
	mean	391 (121)	-	259 (64)	-	66
Wrentham	I	1069 (157)	a,b	515 (83)	a	48
	J	315 (210)	a	81 (112)	b	26
	mean	692 (131)	-	298 (70)	-	43

[1]Values are lsmeans and standard errors from the mixed model ANOVA, with site and fields nested in sites as fixed effects (n=29). Mean separations were determined with a Tukey-Kramer method in SAS (α=0.05). Values with the same letter in a column indicate they were not significantly different.

[2]Percentage of seed loss was calculated as a ratio of viable and total seed lost at harvest.

Table 5-2. Summary of results from the burial trials conducted in Warner and Ellerslie in 2002 and 2004. Estimates from regression analysis of viability of safflower seeds for intercept (*a*) and rate of decline parameter (*b*).[1]

Year	Site	Transgene	Depth (cm)	Parameter estimates (SE) *a*	*b*		EX_{50} DAP[2]	EX_{99} DAP	EX_{50} DP[2]	EX_{99} DP	Frequency of viable seed[3] Spring (May 1)	Fall (Sep. 1)
2002	Warner		0	0.9802 (0.0040)	0.0064 (0.0001)	a	104.5 (1.64)	711.7 (10.99)	19-Mar-2003	15-Nov-2004	0.380 (0.0055)	0.172 (0.0046)
			2	0.9891 (0.0057)	0.0256 (0.0009)	b	26.7 (0.89)	179.6 (6.06)	31-Dec-2002	2-Jun-2003	0.023 (0.0029)	0.001 (0.0002)
			15	0.9908 (0.0056)	0.0267 (0.0009)	a,b	25.6 (0.90)	172.4 (6.14)	30-Dec-2002	26-May-2003	0.020 (0.0027)	0.001 (0.0002)
2002	Ellerlsie		0	0.9833 (0.0034)	0.0057 (0.0001)	c	119.4 (1.84)	810.2 (12.34)	3-Apr-2003	22-Feb-2005	0.428 (0.0054)	0.213 (0.0049)
			2	0.9773 (0.0058)	0.0154 (0.0003)	d	43.6 (0.98)	298.4 (6.51)	17-Jan-2003	29-Sep-2003	0.102 (0.0050)	0.015 (0.0014)
			15	0.9810 (0.0057)	0.0184 (0.0005)	d	36.6 (0.93)	249.0 (6.22)	10-Jan-2003	11-Aug-2003	0.065 (0.0044)	0.007 (0.0008)
2004	Warner		0	0.9565 (0.0057)	0.0072 (0.0001)	e,h	89.4 (1.66)	628.6 (11.13)	31-Jan-2005	24-Jul-2006	0.261 (0.0060)	0.107 (0.0041)
			2	0.9624 (0.0073)	0.0262 (0.0013)	f,g	24.9 (1.26)	174.0 (8.91)	27-Nov-2004	25-Apr-2005	0.009 (0.0021)	0.000 (0.0001)
			15	0.9584 (0.0072)	0.0220 (0.0008)	g,h	29.6 (1.14)	207.6 (7.97)	2-Dec-2004	29-May-2005	0.019 (0.0028)	0.001 (0.0003)
2004	Ellerslie	Present	0	0.9262 (0.0071)	0.0070 (0.0001)	i	87.8 (1.71)	644.8 (11.39)	29-Jan-2005	9-Aug-2006	0.263 (0.0058)	0.111 (0.0041)
			2	0.9154 (0.0088)	0.0160 (0.0004)	j	37.8 (1.14)	282.3 (7.80)	10-Dec-2004	12-Aug-2005	0.052 (0.0041)	0.007 (0.0010)
			15	0.9174 (0.0088)	0.0187 (0.0007)	k	32.4 (1.19)	241.3 (8.46)	5-Dec-2004	2-Jul-2005	0.032 (0.0038)	0.003 (0.0006)
		Absent	0	0.9874 (0.0035)	0.0067 (0.0001)	i	101.2 (1.76)	682.8 (11.76)	12-Feb-2005	16-Sep-2006	0.296 (0.0061)	0.129 (0.0045)
			2	0.9945 (0.0047)	0.0198 (0.0007)	j	34.6 (1.16)	231.7 (7.86)	7-Dec-2004	22-Jun-2005	0.028 (0.0034)	0.002 (0.0005)
			15	0.9954 (0.0047)	0.0207 (0.0008)	k	33.3 (1.22)	222.7 (8.24)	6-Dec-2004	13-Jun-2005	0.025 (0.0034)	0.002 (0.0004)

[1]All values shown are lsmeans (SE, standard errors) from the mixed model analysis (n=72). The values for *a* and *b* were estimated during regression analysis and used extrapolate estimates for days and date after planting for both EX_{50} and EX_{99}, and the mean frequency of viable safflower seeds in spring and fall. Mean separations were determined with a Tukey-Kramer method in SAS (α=0.05). Values with the same letter in a column indicate they were not significantly different.

[2]Days after planting (DAP); Date after planting (DP)

[3]Mean frequency of viable safflower seeds in spring (May 1) and fall (September 1) after trial insertion. May 1 and September 1 of 2003 were 147 and 270 days after inserting the trial in the fall of 2002 and 179 and 302 days after insertion in the fall of 2004.

Table 5-3. Mean density of volunteer safflower plants and growth stage in fields cropped to safflower in 2004 and surveyed during the growing season of 2005.[1]

Date (Relative date)[2]			May 5 (34)		Jun. 16 (76)		Jul. 13 (103)		Aug. 17 (138)		Sept. 9 (161)	
Warner GDD			82.0		328.2		624.6		1045.3		1266.9	
Wrentham GDD			81.1		363.7		651.0		1078.2		1320.8	
Site	Field	Crop[3]	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH
Warner	A	Wheat	0.037 (0.35) a▼■	10.0	1.785 (0.75) a●	11.0	1.64 (1.83) a	53.0	1.88 (0.63) a	83.0	0.33 (0.00)	99.0[4]
	B	Fallow	1.732 (0.29) b▼	10.0	2.65 (0.75) a●	11.0	0.05 (1.83) a	26.5	0.00 (0.63) a●	-	0.00 (0.00)	-
	C	Barley	0.087 (0.35) a,c▼■	10.0	3.3675 (0.75) a●	11.0	0.44 (1.83) a	39.8	0.65 (0.63) a	15.0	0.00 (0.00)	-
Wrentham	G	Fallow	0.071 (0.50) a,c	10.0	17.037 (1.06) b▼	11.0	7.21 (1.83) b,c●	53.0	0.00 (1.27) a	-	0.00 (0.00)	-
	H	Fallow	0.029 (0.50) a,c	10.0	9.275 (1.06) c▼	11.0	1.96 (1.83) a,c●	53.0	0.00 (0.90) a●	-	0.00 (0.00)	-
Sample size			18	18	16	16	20	20	15	15	5	5

[1]All values shown are lsmeans (SE, standard errors) from the mixed model analysis. Mean separations were determined with LSD (α=0.05) and values with the same letter in a column indicate they were not significantly different. Control or field operations prior to sampling are indicated with symbols: ▼ preseed /first herbicide application; ■ seeding complete; ● incrop /subsequent herbicide application(s).

[2]Relative date and cumulative daily growing degree days (GDD) were calculated from April 1, 2004 which is early spring in southern Alberta. The GDD values were calculated above a base temperature of 5C° from daily air temperature values provided by Environment Canada weather stations at Milk River and Wrentham which were the closest available for Warner and Wrentham, respectively.

[3]Fallow fields were treated with herbicides throughout the growing season to control weeds.

[4]Indicate volunteers set seed at the time of harvest. Samples were collected and used in a comparison with safflower crop plants from a separate field at the same farm site (results in Table 5-5).

Table 5-4. Mean number of safflower plants and growth stage in fields cropped to safflower in 2005 and surveyed during the growing season of 2006.[1]

Date (Relative date)[2]			Apr. 19 (18)		May 4 (33)		May 24 (53)		Jun. 15 (75)		Jul. 10 (100)		Aug. 1 (122)	
Warner GDD			25.5		63.0		233.9		412.1		729.2		1054.0	
Wrentham GDD			34.5		83.6		278.4		475.1		796.8		1121.6	
Site	Field	Crop	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH	Safflower Plants m^{-2} (SE)	Stage BBCH
Warner	D	Fallow	7.29 (3.05) a▼	10.0	6.19 (1.5) a	12.0	1.12 (0.32) a●	12.0	2.00 (0.52) a●	30.0	0.39 (0.16) a	14.0	0.00 (0.13) a	-
	E	Barley	10.91 (3.05) a▼	10.0	0.00 (1.5) b■●	-	1.35 (0.32) a	12.0	0.32 (0.52) b,c	16.0	0.31 (0.14) a	24.0	0.18 (0.10) a	87.0[3]
	F	Barley	2.26 (3.05) a▼	10.0	0.00 (1.5) b■●	-	1.07 (0.32) a	12.0	0.03 (0.74) b,c	13.0	0.02 (0.03) a	7.5	0.05 (0.13) a	87.0[3]
Wrentham	I	Wheat	10.35 (3.05) a	10.0	6.95 (1.5) a	12.0	0.00 (0.32) b▼■	-	1.70 (0.52) a,c●	14.0	0.00 (0.32) a	-	0.00 (0.18) a	-
	J	Wheat	2.87 (3.05) a	10.0	6.92 (1.5) a	12.0	0.00 (0.32) b▼■	-	1.42 (0.52) a,c●	14.0	0.00 (0.32) a	-	0.00 (0.18) a	-
Sample size			20	20	20	20	20	20	18	18	15	15	9	9

[1]All values shown are lsmeans (SE, standard errors) from the mixed model analysis. Mean separations were determined with LSD (α=0.05). Values with the same letter in a column indicate they were not significantly different. Control or field operations prior to sampling are indicated with symbols: ▼ preseed /first herbicide application; ■ seeding complete; ● incrop /subsequent herbicide application(s).

[2]Relative date and cumulative daily growing degree days (GDD) were calculated from April 1, 2004 which is early spring in southern Alberta. The GDD values were calculated above a base temperature of 5C° from daily air temperature values provided by Environment Canada weather stations at Milk River and Wrentham, which were the closest available for Warner and Wrentham, respectively.

[3]Indicates volunteers set seed at the time of harvest. Samples were collected and used in a comparison with safflower crop plants from a separate field at the same farm site (results in Table 5-5).

Table 5-5. Comparison of volunteer and crop safflower phenotypic characteristics at time of harvest at Warner, in 2005 and 2006.[1]

Field[2]	Survey year	Crop[3]	Sample size[4]	Plant height	Heads	Branches	Seeds	TKW[5]	Biomass	Total seed	Viable seed	Harvest index[6]	Germination[7]
				cm	plant^{-2}	plant^{-2}	head^{-2}	g	g	plant^{-2}	plant^{-2}		%
D	2005	Safflower	19	64.0 (2.0)	11.8 (2.2)	12.4 (2.2)	18.6 (1.8)	26.2 (1.8)	23.6 (4.0)	235.6 (66.8)	186.1 (52.8)	8.4 (1.5)	79.1 (5.1)
A	2005	Wheat	306	35.0 (0.6)	1.5 (0.2)	1.7 (0.2)	4.7 (0.4)	14.7 (0.9)	1.3 (0.3)	7.9 (4.6)	4.1 (3.6)	5.1 (0.4)	51.6 (4.5)
-	2006	Safflower	34	73.6 (7.8)	7.1 (1.0)	8.8 (1.1)	20.8 (1.4)	30.9 (2.8)	17.6 (3.0)	158.9 (30.7)	157.3 (30.4)	8.7 (0.7)	99.0 (0.3)
E & F	2006	Barley	148	33.6 (12.6)	1.1 (0.3)	1.4 (0.3)	0.8 (0.7)	2.2 (2.0)	1.0 (0.7)	2.2 (1.8)	1.2 (1.0)	0.6 (0.5)	49.8 (5.3)

[1]All values are lsmeans and standard errors from the mixed model ANOVA, except for % germination which is a mean and standard error from the raw data. All comparisons of safflower volunteer and crop plant characteristics (one tailed tests) were highly significant ($p < 0.001$), except for plant height in 2006 ($p = 0.067$) and harvest index in 2005 ($p = 0.0013$).

[2]Field designations correspond to Tables 5-3 and 5-4.

[3]Crop growing in each field when the safflower samples were harvested.

[4]Number of plants sampled from each field.

[5]Thousand kernel weight (TKW)

[6]Harvest index was calculated for each plant as the ratio of total seed and biomass.

[7]Germination percentages and standard errors were calculated from raw data. Germination means and standard errors for volunteers from fields E and F were estimated from bulked seeds.

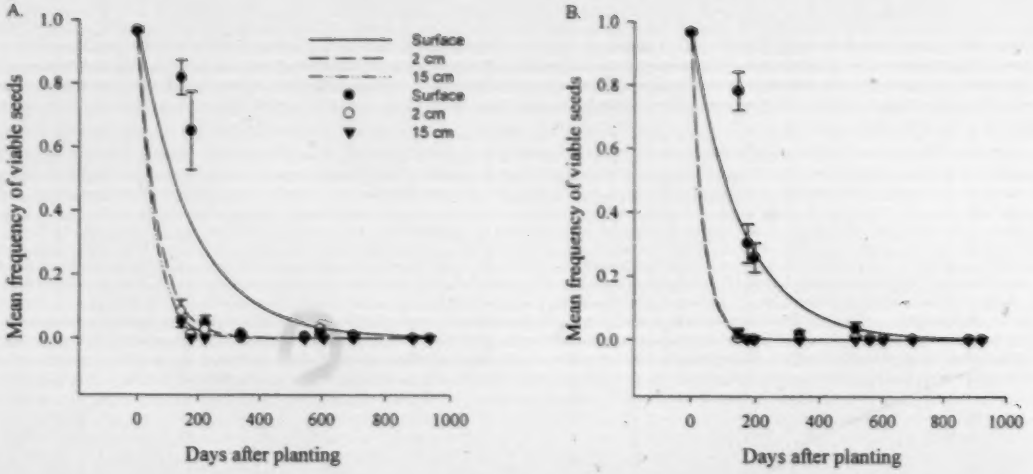

Figure 5-1. Safflower seed survival curves for Ellerslie (A) and Warner (B) in 2002. Lines were generated by non-linear regression fitting equation 1 to the seed viability data for all three depths. The symbols are the observed mean frequency of viable safflower seeds with bars indicating one standard error of the mean (n=72). Note: several of the standard error bars are obscured by symbols.

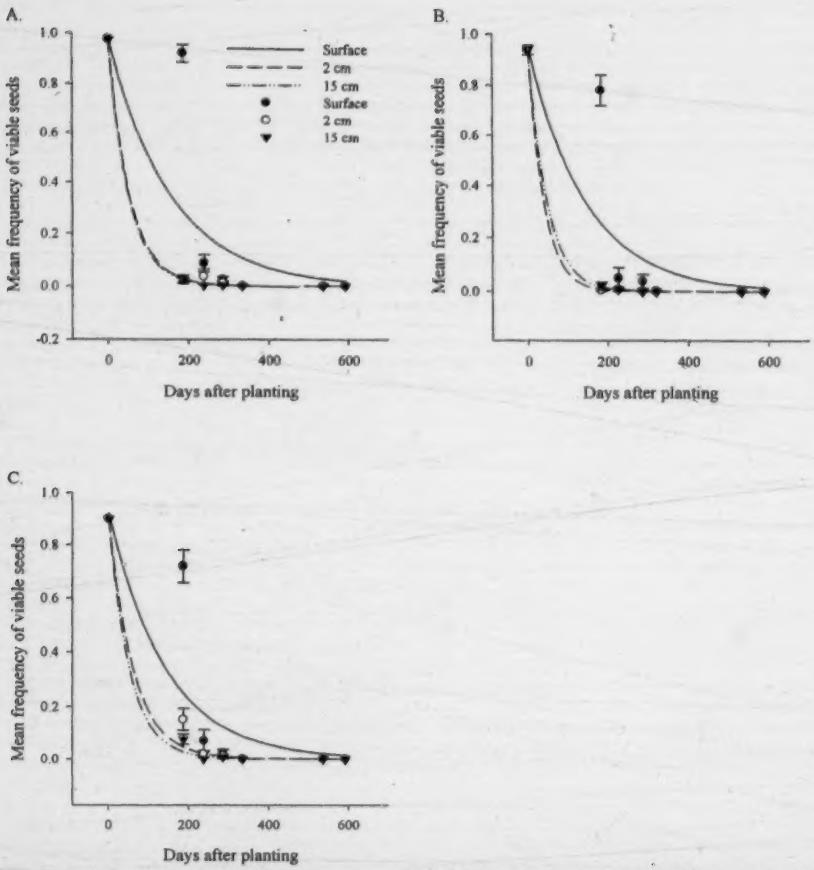

Figure 5-2. The seed survival curves in 2004 for transgenic safflower at Ellerslie (A), non-transgenic safflower at Ellerslie (B) and both genotypes at Warner (C). Lines were generated by non-linear regression fitting equation 1 to the seed viability data for all three depths. The symbols are the observed mean frequency of viable safflower seeds with bars indicating one standard error of the mean (n=72). Note: several of the standard error bars are obscured by symbols.

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Chapter 6: Biosafety of plant molecular farming: herbicide and cultural methods to control transgenic safflower (*Carthamus tinctorius* L.) volunteers in barley and canola

INTRODUCTION

An oilseed crop, safflower (*Carthamus tinctorius* L.), has been transformed with a construct to confer resistance to a broad-spectrum herbicide (glufosinate) and seed-specific production of high-value proteins using plant molecular farming (PMF). In Canada, deregulated transgenic crop plants with production traits such as herbicide and insect resistance, have been released and grown without confinement or segregation from commodity crops. It is likely that PMF crops and products will require segregation from food and feed crops similar to breeder (pedigreed) seed production. Management practices have been established for confined release field trials, which in Canada are conducted under strict regulations including but not limited too: isolation from cross-compatible crops/wild relatives, monitoring and documentation of seed movement and trial operations, third party inspections of field sites and documentation, and post-harvest land restrictions on follow crops and cleaning all equipment used on the site. At this time, regulations and thresholds of PMF seed in food/feed and plant products have not been established by the Canadian Food Inspection Agency (CFIA) and no PMF crop has been permitted for commercial production in Canada.

A concern of field scale production of safflower for PMF is the presence and control of transgenic safflower volunteers in post-harvest years. If volunteers occur in follow

commodity crops, they could flower and pollinate neighbouring non-transgenic safflower, they could set seed, and either recharge the seed bank and/or become admixed (commingled) with harvested commodity crop. In Nebraska (United States), volunteers of a corn (*Zea mays* L.), expressing a protein for PMF, from a small plot experiment the previous year, was admixed with commodity soybeans. The corn volunteers were inadvertently harvested with the soybean (*Glycine max* L.). The soybeans were then shipped and stored with 17.5 million litres of other soybeans that were then ordered destroyed by the US regulators (Ellstrand, 2003) at a cost to the company Prodigene Inc. of $2.7 million US (Stewart and Knight, 2005).

Anecdotal evidence from the literature suggests that safflower does not persist after cultivation and, if it volunteers, it is only in the year following production (Smith, 1996). In the United States, safflower has been documented as a volunteered in the agroecosystem in Arizona, California, Colorado, Idaho, Iowa, Illinois, Kansas, Massachusetts, Montana, Nebraska, New Mexico, North Dakota, Ohio Oregon, Utah and Washington (Berville et al., 2005; Keil, 2006; McPherson et al., 2004). In Canada safflower has been reported as escaped cultivation in Alberta and British Columbia. Safflower has not been identified as a volunteer in the three large scale weed surveys of cultivated land on the Canadian Prairie Provinces (Alberta, Saskatchewan, and Manitoba) conducted to date (Leeson et al., 2005), but the Alberta portion of the surveys has shown other crop volunteer to occur at high frequency (Leeson et al., 2001). The rank of weeds based on the frequency of occurrence in Alberta fields surveyed included, several volunteer crop species in the top 100: canola (*Brassica napus* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), alfalfa (*Medicago sativa* L.), oats (*Avena*

sativa L.), flax (*Linum usitatissimum* L.), chickpea (*Cicer arietinum* L.), pea (*Pisum sativum* L.), and rye (*Secale cereale* L.). Safflower is a minor use crop in southern Alberta and Saskatchewan, with annual production between 810 and 2,025 hectares, primarily for birdseed (Mundel et al., 2004). This low annual acreage in a few fields in Alberta makes its detection unlikely in random weed surveys, even if volunteers occur.

Safflower is a poor competitor with weeds, especially early in the season in the rosette stage (3-4 weeks) before bolting (Blackshaw et al., 1990a; Blackshaw et al., 1990b; Smith, 1996). In Canada, few herbicides have been registered for weed control in safflower, and these are limited to the pre-plant, soil-applied group 3 herbicides Edge (ethalfluralin) and Rival (trifluralin), and a single foliar applied group 1 herbicide, Poast Ultra (Sethoxydim) (Ali, 2003; Ali, 2003; Blackshaw et al., 1990a; Blackshaw et al., 1990b). Thus, these herbicides would not be appropriate in years following PMF with safflower to control volunteers. Annual weeds of the Asteraeceae are controlled with various herbicides with different modes of action (Ali, 2003; Mallory-Smith and Retzinger, 2003), during chemical fallow or in-crop applications. However, herbicides have not been tested (to the authors' knowledge) or registered for safflower volunteer control in follow years.

In addition to herbicides', several agronomic practices may limit safflower volunteer persistence and seed production in follow years. The effectiveness of an integrated weed management (IWM) approach to limit weed impacts on crops and limit weed seed production, has been studied for wheat, barley and canola on the Canadian prairies (O'Donovan et al., 2007). These include practices to increase crop competition, and chemical and mechanical control of weeds. Management decisions are based on scouting

fields to identify weeds and their stage and density. An IWM strategy to limit safflower volunteer survivorship and reproduction would require knowledge of both herbicide efficacy and follow crop management influences on safflower biology.

We conducted two studies to investigate safflower volunteer fecundity and control. In the greenhouse, I screened various herbicides over a range of doses, to identify their utility for safflower control. Second, I performed field studies to assess the admixture potential of safflower, in two potential follow crops, barley and canola. The survivorship and fecundity (viable seed production) of volunteer transgenic safflower intended for PMF were quantified for both barley and canola crops under various management regimes. I evaluated the influence of barley seeding rates and types of herbicides on safflower control. I included four canola systems, each with specific variety and herbicide, to determine if systems differed in their influence on safflower control. The admixture of safflower seed and its viability in harvested barley and canola seed were quantified. These studies are a component of a larger project intended to examine biosafety and risk mitigation of PMF with safflower.

RESULTS

Dose-response

Greenhouse experiments were used to determine the relative effectiveness of a broad range of herbicides (Tab. 1) on safflower growth and survival, and to determine if there were differences in herbicide tolerance between transgenic and non-transgenic safflower plants. With the exception of the herbicide glufosinate, transgenic and non-transgenic safflower responded similar to all herbicides, as determined by comparison of Akaike

Information Criterion Corrected (AICC) (Tab. A1 and Figs. 6-1 to 6-3) (Crossa et al., 2004; Littell et al., 2006). Transgenic safflower biomass response to glufosinate was linear, indicative of the expected tolerance to this herbicide (Fig. 6-1). Non-transgenic safflower biomass decreased exponentially, with increased dosage of glufosinate, indicative of susceptibility to this herbicide (Fig. 6-1). Safflower biomass response to increased dosages of quizalofop-p-ethyl (Assure II), sethoxydim + ethametsulfuron-methyl (Poast Ultra + Muster) and ethametsulfuron-methyl (Muster) was linear indicating little or no reduction with increased dosage (Fig. 6-2 and Tab. A6-1). Safflower biomass decreased exponentially with increased herbicide dosage of all other herbicides (Fig. 6-3 and Tab. A6-1).

The relative effectiveness of herbicides for controlling safflower, can be evaluated by comparing the ED_{50}, the estimated effective dose of herbicide required to reduce safflower biomass by 50%, here expressed as a proportion of the recommended rate of herbicide (Ali, 2003) (Fig. 6-1 to 6-3 and Tab. A6-1). Glufosinate (Liberty) had a significant effect on non-transgenic safflower biomass, with an ED_{50} of 0.21, but as expected no effect on transgenic safflower as expected (Tab. A6-1 and Fig. 6-1, $p=0.0007$). Contrasts of the ED_{50} values for both safflower genotypes for all of the other herbicides were not different ($p > 0.05$).

Three herbicide treatments, ethametsulfuron-methyl (Muster) alone, quizalofop-p-ethyl (Assure II) and sethoxydim/ethametsulfuron-methyl (Poast Ultra/Muster) had little effect on safflower (Tab. A6-1 and Fig. 6-2, $ED_{50} > 2.0$). Ethametsulfuron-methyl is used to control wild mustard and other weeds in canola and quizalofop-p-ethyl is used to control grass weeds and volunteer cereals in canola. Both of these herbicides have limited

control spectrum for broadleaved plants (Ali, 2003). Sethoxydim is registered for grass weed control in safflower, and did not reduce safflower biomass (Fig. 6-2) (Ali, 2003; Blackshaw et al., 1990a). All of the other herbicides were highly effective at controlling safflower with ED_{50} values ranging from 0.12 to 0.25 (Table A6-1 and Figs. 6-2 and 6-3).

Admixture field studies

Field trials were conducted at three locations in each of two growing seasons to quantify safflower volunteerism, survival, fecundity and admixture in cereal and broadleaf follow crops. Pre-planting, safflower seeds were spread over the soil surface. Barley and canola were planted and grown using conventional management practices. Treatments for barley included three seeding rates and three commonly applied in-crop herbicide combinations. Treatments for canola systems included recommended seeding rates and commonly applied in-crop herbicides to this crop, in the Canadian growing region.

Weather patterns during admixture trial growing season

Precipitation and air temperature varied among the sites and years (Figs. 6-4 and 6-5). Foremost in 2004, was the hottest and driest site with mean monthly rain fall below 50 mm throughout the growing season, and mean, minimum and maximum monthly temperatures above all the other sites in both years. Ellerslie in 2004 and 2005, was the wettest and coolest sites with mean monthly rain fall above 50 mm throughout the growing season except for June in 2004. The mean monthly temperatures at Ellerslie in both years were below those of the other sites and years. Lethbridge experienced an unusually high rainfall in the month of June and September in 2005 relative to the 98 year average, and rainfall was below the long term average for the months of May and June. The mean monthly temperatures at Lethbridge were similar to the 98 year average in all

months. The rainfall pattern in Warner in 2004 was similar to the 12 year average, but in 2005 the pattern was similar to Lethbridge. The mean monthly temperatures in Warner were similar to the long term 12 year average (Figs. 6-4 and 6-5). Safflower's area of adaptation is the hot and dry southern portion of the province. Canola is usually grown in cooler and wetter conditions and barley can be grown in either area.

Barley admixture

In-crop herbicides significantly reduced volunteer safflower biomass, plant density, total seeds m^{-2}, and viability of safflower seed admixed with harvested barley. They did not reduce barley yield and biomass, percent admixture of safflower seeds in harvested barley grain (w/w %) and safflower viability (Tabs. 2). Herbicidal control was not significantly different between sites and years, except total admixed safflower $m\text{-}^{2}$ in harvested barley differed among the sites (significant site x herbicide treatment interaction). All herbicides used provided adequate control (70 to 98%) of safflower relative to untreated controls 2 and 4 weeks after treatment (WAT) and would be acceptable in conventional crops ($p<0.00005$; Tab. A6-2). Control increased with time as crop competition contributed to safflower mortality. Safflower control 6 WAT at all sites and years was 93 to 97% (Tab. 2). Barley seeding rates did not significantly increase safflower control 2, 4, and 6 WAT. All three in-crop herbicides decreased safflower survivorship early in the growing season (Tab. 2).

At the time of barley harvest, safflower volunteer density and biomass were low in herbicide treated and untreated plots, in all sites and years (Tabs. 2). No safflower plants or biomass were recovered from Ellerslie in 2004. Thus, this site/year was excluded from the ANOVA. Herbicides usually significantly reduced safflower biomass and density in

all sites and years relative to untreated controls, but herbicide treatments were not significantly different.

Safflower seed admixed with harvested barley from all treatments and untreated controls was low in all sites and years (Tab. 2). No safflower seeds were recovered from any harvested barley plots at Ellerslie in 2004 and 2005. No safflower seeds were recovered from herbicide treated plots at Lethbridge and Warner in 2005. Few safflower seeds were recovered from harvested barley in 2005 but none were viable (Tab. 2). When safflower seeds were found admixed with harvested barley, less than 10% of the seeds were viable at all sites from herbicide treated plots (Tab. 2).

Barley seeding rates did not significantly impact safflower emergence (data not shown), suggesting barley competition after emergence limited safflower admixture. Barley is a competitive crop and produced relatively consistent yields and biomass, under a variety of weather conditions at all sites and years. Barley plant competition effectively limited safflower survival and fecundity as indicated by the low density of safflower plants, biomass and seed recovered from barley not treated with herbicides at all sites and years. Herbicides further reduced safflower seed survival, and viable seed admixture (fecundity) in most cases.

Canola admixture

The survival, vigour and fecundity of safflower volunteers in four canola systems (Liberty Link, Roundup Ready, Clearfield, and conventional) was measured with visual control ratings after herbicide application, safflower biomass and density at harvest and the total number and viability of safflower seeds admixed with harvested canola seeds (Tab. 3). Safflower growth early in the season was influenced by all herbicides except

glufosinate. At harvest safflower biomass, density and admixture with harvested canola were marginally reduced by imazethapyr/imazamox and strongly reduced by glyphosate but not the other herbicides (Tab. 3). Canola biomass and yield, and safflower biomass, density and seed admixture at harvest were not significantly different between site and year.

Safflower response to herbicides applied in-crop to each canola system, correlated with the greenhouse trials. Application of glufosinate and sethoxydim/ethametsulfuron-methyl on canola did not adequately control safflower volunteer growth 6 WAT (0% and 0 to 30% control, respectively). Imazethapyr/imazamox applied to Clearfield canola suppressed safflower volunteers 6 WAT (67% control; $p=0.0022$). Application of glyphosate on canola provided excellent control of safflower volunteers 6 WAT (99.5% control; $p<0.0005$). Safflower emergence was not significantly different prior to herbicide treatment, but canola emergence was significant at Warner in 2004 and 2005 ($p = 0.0325$ and 0.0218, respectively; data not shown). However, these differences were not correlated with treatment differences. Although highly variable among sites and years, safflower biomass, density and admixture with harvested canola were generally correlated with efficacy of herbicides relative to untreated controls early in the growing season.

In all the systems in 2004 and 2005, canola biomass and yield were not significantly influenced by herbicides, but a small improvement in canola yield did occur for Clearfield ($p=0.03$) (Tab. 3). Safflower volunteer biomass, density and admixture with harvested canola, were not reduced by herbicides applied in-crop in the Liberty Link and conventional canola systems. Safflower volunteer biomass and density were reduced in

the Clearfield system by application of imazethapyr/imazamox (p=0.0155 and 0.04025, respectively). However, safflower admixture with harvested canola was not reduced in this canola system. Safflower volunteer biomass, density, and both the percentage (w/w %) and total safflower seed admixed with harvested canola was significantly reduced by application of glyphosate in the Roundup Ready system (p= 0.01695, 0.00525, 0.0355 and 0.02245, respectively). However, safflower seed viability (% of total admixed seeds) was not significantly reduced by glyphosate application.

In addition to herbicides, safflower volunteer survival and fecundity in each canola system was influenced by canola competition and weather. Canola yields and biomass were high at the northern most location, Ellerslie, where it is more adapted than safflower (data not shown). Conversely, no safflower plants were recovered from Ellerslie in 2004, and in 2005 some safflower plants survived to harvest, but produced limited biomass. These plants did not mature to seed set, and admixture with the harvested canola was not detected. Safflower volunteers were more vigorous in the southern drier sites where it is more adapted. In these sites, safflower reached maturity and set some seed in all years and sites. The zero values at the Ellerslie sites prevented ANOVA but reflect the negative influence of wet and cool weather and competitive canola crops on safflower volunteers.

DISCUSSION

The field studies here have confirmed that in the Canadian agroecosystem, safflower can volunteer in the follow crops barley and canola. Although, safflower was a poor competitor with barley, it may survive herbicide control to produce seed. However, all of the herbicides applied in-crop to barley, reduced safflower volunteers' survival and fecundity relative to untreated controls. In the absence of herbicides, safflower was

competitive with canola under dry and hot weather conditions, but when weather was warmer and cooler, safflower was less competitive with this crop. Volunteer safflower survival and fecundity in canola was significantly reduced by glyphosate, and suppressed with imazethapyr/imazamox, but was not significantly reduced by glufosinate and sethoxydim/ethametsulfuron-methyl relative to untreated controls. Thus, seed from safflower volunteers in follow years can become admixed with harvested barley and canola seed. Several herbicides have utility for control of safflower volunteers in follow crops and may be used as part of a follow year management strategy to reduce admixture. Transgenic and non-transgenic safflower response to different herbicides was similar except for glufosinate, as would be expected.

Safflower takes longer to reach maturity than barley and canola, but volunteer safflower plants produced some viable seed in these follow crops. The variety of safflower considered for PMF production is 'Centennial', which requires *ca.* 126 days to mature, whereas, canola and malting barley mature after *ca.* 109 and 98 days, respectively, in Alberta. Additionally, barley is known to have a wide range of adaptation in Alberta and is a highly competitive crop, relative to other crops like wheat and canola (O'Donovan et al., 2007). Volunteer safflower in barley did not produce large amounts of mature seed at the time of harvest, and this would limit admixture of safflower in this crop. Canola is not a competitive crop in southern Alberta where safflower is productive, even with excellent herbicide control. Safflower volunteers in canola may survive and produce viable seeds, some of which would be admixed with the harvested seed. An IWM strategy to limit safflower seed production and admixture in subsequent growing seasons should incorporate barley or other cereal as a follow crop, rather than canola.

However, the recovery of some safflower seed and biomass from both barley and canola suggest that safflower can volunteer, flower, set seed and become admixed during harvest.

This study documented and quantified the presence of flowering safflower volunteers in barley and canola follow crops. This may contribute to non-viable, but still detectable transgenes or transgenic proteins in harvested crops. Transgenic safflower seed lost at harvest may contribute to the seed bank and continued gene flow, in subsequent years. Transgenic safflower volunteers could potentially cross-pollinate conventional safflower in neighbouring fields intended for conventional markets. Although, safflower seed admixture with harvested barley and canola was low, it could be moved great distances and either consumed or replanted.

Regulators and the public may demand thresholds for contamination of food and feed with PMF safflower, which could be set to zero, as is currently designated in the USA for avidin and trypsin produced in corn (Howard, 2005). Such stringent thresholds would preclude cropping the year following PMF production. Thus, the year following PMF safflower production the land would need to be either chemical fallow or a green manure crop. With less stringent thresholds, a competitive cereal crop with careful herbicide choice and applications could be grown in the follow year. This research will be used to formulate a best management practices for commercial field scale plant molecular farming with safflower.

MATERIALS AND METHODS

Dose-response

The response of transgenic and non-transgenic safflower to different herbicides intended for safflower volunteer control were evaluated under controlled conditions. Experiments were conducted in glasshouse conditions at the University of Alberta, Edmonton, Alberta, Canada, from 2004 to 2006. Transgenic and non-transgenic seeds were planted in trays with six cells each (1 L per cell) containing soil-less vermiculite-peat mixture (Metro-Mix 290, The Scott's Company, 14111 Scottslawn Rd., Marysville, Ohio 43041). Each tray had three pairs of each transgenic and non-transgenic genotype. Twelve seeds per cell were planted, and after emergence, were thinned to eight plants. For each herbicide, paired cells of transgenic and non-transgenic plants were randomized in each of three blocks (RCBD). Plants were exposed to natural light supplemented to 16-h with 400 watt high pressure sodium high intensity discharge bulbs, and maintained at 21/18 °C day/night temperature. Plants were watered as required and fertilized biweekly with complete 20-20-20 plus at a concentration of 200 ppm.

Thirteen herbicides were (Tab. 1) applied 0.25, 0.5, 1, 1.5 and 2 times the recommended field rates (Ali, 2003) when safflower plants had two to six true leaves using a custom built track sprayer using an Air Bubblejet nozzle, calibrated to apply 100 L ha^{-1} at 200 KPa. After herbicide application, trays were returned to the greenhouse and irrigated from above as required (at least 24 h after application). Twenty one days after herbicide application, plants were counted and cut at the soil surface, dried in paper bags at 60 C° for five days and dry weight was determined. Each herbicide dose-response experiment had three replicates and was repeated three times (replication in time).

Admixture field studies

Field experiments were conducted during the growing season of 2004 and 2005 at three locations in each year in Alberta, Canada. One trial site was located at the Ellerslie Research Farm in central Alberta, on black chernozemic soil in 2004 and 2005. The other three trial sites were located in southern Alberta on brown chernozemic soil near the towns of Warner, in 2004 and 2005, Foremost, in 2004 and Lethbridge in 2005. Soil samples were analyzed from each site and year (Tab. A6).

The transgenic safflower cv. 'Centennial' intended for PMF, was resistant to the broad-spectrum herbicide glufosinate, but susceptible to herbicides similar to conventional safflower (see results of the dose response experiment). To reduce expense and the risk of potential movement of transgenic seeds, safflower used in the barley trials was non-transgenic but the same variety as the transgenic line (safflower cv. 'Centennial'). However, because one of the canola systems included a glufosinate resistant variety, these trials were conducted with glufosinate resistant safflower. To comply with regulations established by the Canadian Food Inspection Agency (Plant Biosafety Office), the non-transgenic and transgenic trials were grown at least 800 m from one another, and 1600 m from safflower production intended for food or feed ([CFIA] Canadian Food Inspection Agency, 2006).

At the Ellerslie Research Farm, site fertilizer was applied at the time of seeding. Nitrogen (80 Kg ha^{-1}) was side banded, and phosphorus (30 Kg ha^{-1}), potassium (60 Kg ha^{-1}) and sulphur (20 Kg ha^{-1}) placed with the seed. At Foremost and Warner, nitrogen was banded prior to seeding using a Barton low disturbance openers at a depth of 7 to 8 cm and was applied at 60 Kg ha^{-1}. Phosphorus was placed with the seed at 20 Kg ha^{-1} at

the time of seeding. At the Lethbridge site, nitrogen fertilizer (46-0-0) was broadcast at a rate of 70 Kg ha^{-1} and soil incorporated with cultivation followed by harrow at *ca.* 7 cm, and phosphorus was placed with the seed at 20 Kg ha^{-1} at the time of seeding.

Glyphosate (810 g ai ha^{-1}) was used to control vegetation prior to seeding. To simulate safflower volunteer densities, seeds were spread over the soil surface by hand at a density approximately 50 seeds m^{-2}, half to one third of the recommended seeding rate for safflower (Mundel et al., 2004). A previous study suggested this density was higher than viable seeds lost during harvest and surviving the Canadian prairie winter (McPherson et al., in preparation). Immediately following the safflower seed spreading, canola or barley crops were seeded. Germination of both genotypes (transgenic and non-transgenic) of safflower seed used was determined to be 96% for both in 2004, and 99% and 75.5% for the transgenic and non-transgenic in 2005, respectively.

At the Ellerslie Research Farm, barley and canola were seeded with a reduced disturbance custom Fabro air seeder, equipped with atom jet double shoot openers spaced at 20.32 cm at the centre. At Warner, Foremost and Lethbridge, the seeding was conducted with a self propelled small plot seeder with John Deere no-till disc openers and row spacing of 18 cm. The canola was seeded 2.5 cm deep with a seeding rate to attain a target plant stand of 200 plants per m^2. The barley cv.'AC Metcalf' was seeded at 4.4 cm at a rate based on germination tests and 1000 kernel weight for a target plant densities of 150, 200, 250 plants m^{-2}. Seeding of barley and canola at Ellerslie was conducted on May 14, 2004 and May 9 and 10, respectively, in 2005. Seeding of both crops at Warner and Foremost was on May 10, 2004. Seeding of both crops at Warner and Lethbridge was on May 5, 2005. At all sites, the plots were 1.8 m by 10 m in length, with six rows of crop

and one outside row of winter wheat to decrease edge effects and to facilitate weed control between the plots. Plots were trimmed (7.2 to 8.6 m) prior to harvest, at all sites except at Lethbridge where they were mowed throughout the season (7.1 to 7.3 m). Plot lengths were measured at the time of harvest and used to calculate any values involving area (i.e. crop yield and safflower volunteer plants m^{-2}).

Weeds were removed from untreated control plots (no herbicide) by hand throughout the growing season. Herbicides were applied at field rates with a CO_2-pressurized backpack sprayer with low drift Air Bubblejet nozzles at a volume of 100 L ha^{-1}, (Tab. 1, (Ali, 2003). Prior to herbicide application, densities of emerged crop and safflower plants were measured in 2004 with four 0.25 m^2 areas per plot and in 2005 with three 0.25 m^2 areas per plot. Time of herbicide application was based on safflower stage of development. Crop and safflower stages at the time of herbicide application were noted and converted to a universal crop scale (BBCH scale; (Meier, 2001)). In 2004, at the time of herbicide application, the safflower volunteers were at the 2 to 5 leaf stage (BBCH codes 12 to 15); the canola was between the 2 and 5 leaf stage (BBCH codes 12 to 15), and the barley had 2 to 3 tillers (BBCH codes 22 to 23). In 2005, the safflower volunteers were at the 2 to 6 leaf stage (BBCH codes 12 to 16), the canola was between the 6 leaf and stem elongation stage (BBCH codes 16 to 30) and the barley was at the first leaf to three tiller stage (BBCH codes 11 to 23).

Three herbicides commonly used in-crop to control broadleaf weeds in barley were used (thifensulfuron-methyl/tribenuron-methyl at rates of 5/5 g ai ha^{-1}; bromoxynil/MCPA at rates of 280/280 g ai ha^{-1}; and of 2, 4-D at a rate of 394.4 g ai ha^{-1}).

All broadleaf herbicides were tank mixed with fenoxaprop-p-ethyl at a rate of 92.51 g ai ha^{-1} to control grass weeds (Tab. 1).

Each system consisted of a canola variety and a specific herbicide. The systems were Liberty Link Invigor 2663® sprayed with Liberty® (glufosinate ammonium at 400 g ai ha^{-1}), Round-up Ready DKL3455® sprayed with Roundup Transorb® (glyphosate at 810 g ai ha^{-1}), Clearfield 46A76® sprayed with Odyssey® (imazamox and imazethapyr both at 14.70 g ai ha^{-1}), and a conventional non-transgenic variety Quantum II® which was sprayed with Poast Ultra®/Muster® (sethoxydim/ethametsulfuron-methyl at 211.19/ 22.23 g ai ha^{-1}) (Tab. 1).

Post-herbicide data collection and harvest

Control of volunteer safflower was evaluated visually two, four and six weeks after herbicide treatment (WAT) on a scale of 0% (no control) to 100% (complete control relative to untreated control). Above-ground biomass of the crop and safflower volunteers were estimated from three 0.25 m^2 area random biomass samples collected from each plot at the time of harvest. Safflower plant density was counted and biomass collected from each 0.25 m^2 area. The harvested above ground biomass was placed in paper bags and dried at 60 °C for several days. Following dry weight measurements, the safflower plants were threshed by hand and the number of seeds $plant^{-1}$ was determined.

Grain was harvested from the remainder of the barley and canola plots with a Wintersteiger Elite harvester, with the settings adjusted to simulate a commercial scale harvest for each crop. The settings for harvesting barley were: concave with two de-awners (scrubbers) size 12, concave clearance 5 mm in the front and 3 mm in the back, threshing drum speed was 1250 to 1300 rpm, the openings of the upper and lower shaker

sieves were 16 and the cleaning wind force was set to 3/4 (1000 and 1500 rpm). The settings for harvesting canola were: concave without de-awners (scrubbers) size 6, concave clearance 12 mm in the front and 8 mm in the back, threshing drum speed was 950 rpm, the openings of the upper and lower shaker sieves was 10 and the cleaning wind force was set to 1/4 (600 and 800 rpm).

Barley and canola were harvested in 2004 at Warner and Foremost between September 1 and 3, and at Ellerslie on October 1. Barley was harvested in 2005 at Warner and Lethbridge on August 29, and Ellerslie September 6. Canola was harvested in 2005 at Warner and Lethbridge on September 9 and 20, and Ellerslie on September 26. The seed was cleaned with a bench top air screen cleaner (Clipper Office Tester ® Clipper Seed and Grain Conditioning Ltd.) and crop yields were estimated. Volunteer safflower seeds were removed during the cleaning of the canola, but removed by hand sorting from the barley. Safflower seeds from each plot were counted and later tested for viability (see below).

Seed viability testing

Seeds were placed in acrylic germination boxes (24 x 16 x 3.8 cm) (Hoffman Manufacturing, Inc.) between 15 x 23 cm non-toxic white filter paper equivalent to Whatman #1 (Hoffman Manufacturing, Inc.). To imbibe seeds and reduce microbial growth, 14 mL of a 0.2 % solution of Helix XTra ® (thiamethoxam / difenoconazole / metalaxyl-M / fludioxonil) was added to each germination box. Previous experiments with this solution indicated that it did not inhibit germination of safflower seeds (data not shown). The germination trays were stored in the dark at ambient temperature for four to five days. Seeds with a radical emerged were considered viable. Un-germinated seeds

were cut along the pericarp (seed coat) suture line, placed embryo side down in 0.015% tetrazolium chloride and incubated for 2 hours at 30 °C (Porter et al., 1947). Seeds with a positive tetrazolium test were considered viable.

Statistical analysis

Statistics dose-response

A ratio of the safflower biomass, measured for the control and the herbicide treatments for each genotype, was calculated separately for each genotype (transgenic and non-transgenic), herbicide, herbicide dose, and replicate in time to eliminate confounding differences. For each replicate in time the coefficient of variation (CV) was calculated. If the was above 35% the value deviating the furthest from the overall mean was removed from further analysis.

Nonlinear regression analyses with both a linear and log-logistic equations were conducted to model the response of safflower biomass to increasing doses of each herbicide.

$$Y = mx + c \qquad (1)$$

$$Y = ae^{-bx} + d \qquad (2)$$

where y is the proportion of water control as a function of dose (x), c and a are intercept, m is slope, b is the rate of decline and d is the asymptote. These equations and the normal distribution were used to model the error and proportion of water control (~ normal (0, s) in a nonlinear regression mixed model analysis in SAS, using PROC NLMIXED to derive estimates for each parameter and variance (s, σ^2). Data for each herbicide and genotype were regressed with linear and nonlinear equations. The model with the lowest

Akaike Information Criterion Corrected (AICC) value provided by SAS, was used to estimate the parameters and ED_{50} values.

The estimate of the effective herbicide dose that reduced the safflower biomass by 50% (ED_{50}) was conducted with an ESTIMATE statement in SAS equation 3 and 4 and the best fitted model.

$$ED_{50} = \frac{50 - c}{m} \qquad (3)$$

$$ED_{50} = \frac{\ln 50 - (0.5 * d) - \ln a}{-b} \qquad (4)$$

where a and c are intercept, m is slope, b is the rate of decline and d the asymptote.

Statistics admixture field studies

All response variables were subject to a mixed model ANOVA (PROC MIXED) in SAS with years, sites, and blocks (replicates) as random effects, and herbicide treatment and seeding rate as fixed effects (SAS Institute Inc., 2007). Analysis was hampered by zero values and a lack of variance on some occasions; when this occurred, means and standard errors were reported. Square root transformation of several variables improved model fit statistics, but did not alter the magnitude of significance in any case. Thus, all data were analyzed without transformation. Emergence of safflower volunteers was significant prior to herbicide application at Ellerslie 2005, for safflower in glufosinate resistant canola. Canola emergence was significant prior to herbicide application at Warner in 2004 and 2005 for canola varieties resistant to glyphosate (Roundup Transorb) and imazethapyr/imazamox (Odyssey), respectively. However, ANOVA on data collected at harvest, analyzed with the emergence counts as covariates did not provide meaningful results. The canola experiments were designed and randomized separately, by system

because the variety and herbicide are not independent. Thus, each canola system was analyzed separately and variance of herbicide and untreated controls were modeled separately for each ANOVA (Tab. 1). Mean separation and multiple comparisons were conducted with a Tukey-Kramer adjustment ($\alpha = 0.05$) in SAS. A contrast (Estimate statement in SAS) was conducted to compare all of the herbicide treatments to the untreated control (SAS Institute Inc., 2007). Although all of the analysis years and sites were not significant, they were still analyzed and presented separately to demonstrate consistencies, and highlight several instances where safflower responses produced low or zero values.

Table 6-1. Herbicide treatments and rates used for the greenhouse dose-response experiments and for field trials to assess safflower admixture in barley and canola.

Treatment number	Trade name [1]	Common name	Herbicide group [2]	1X Rate [3] g ai ha^{-1}
1	Assure II	Quizalofop-p-ethyl[4]	1	47.4
2	Buctril M / Puma Super	Bromoxynil/ MCPA/ fenoxaprop	6/4/1	280.0/280.0/92.5
3	2, 4-D/Puma Super	2, 4-D ester /fenoxaprop	4/1	394.4/92.5
4	Caliber	2, 4-D B	4	1200.0
5	Liberty	Glufosinate ammonium	10	400.0
6	Lontrel	Clopyralid	4	151.2
7	Muster	Ethametsulfuron-methyl	2	22.2
8	Odyssey	Imazethapyr/imazamox[5]	2	29.4
9	Pardner	Bromoxynil	6	280.0
10	Poast/Muster	Sethoxydim/ethametsulfuron-methyl[5]	1/2	211.2/22.2
11	Refine Extra/Puma Super	Thifensulfuron-methyl /tribenuron-methyl/fenoxaprop	2/1	5.0/5.0/92.5
12	Roundup Transorb	Glyphosate	9	810.0
13	Sencor	Metribuzin	6	345.0

[1]Manufacturer names from (Ali, 2003).
[2]Herbicide groups classified by site of action (Mallory-Smith and Retzinger, 2003).
[3]Recommended rate of herbicide from (Ali, 2003).
[4]Applied with 0.5% v/v Assure Mix.
[5]Applied with 0.5% v/v Merge.

Table 6-2. Production response of barley and transgenic safflower volunteers to herbicide treatments at Ellerslie, Foremost, Lethbridge and Warner in 2004 and 2005.[1] No safflower plants or seeds were recovered from Ellerslie in 2004 and no safflower seeds were recovered from Ellerslie in 2005. No viable safflower seeds were recovered from Ellerslie in either year and Lethbridge and Warner in 2005.

Herbicide treatment	Visual ratings 6 WAT[2]	Barley		Safflower[3]		Safflower[3,4]					
		Yield	Biomass	Biomass	Density[4]	Admixture	Total seeds m^{-2} Site			Viable[5]	
	%	t ha^{-1}	g m^{-2}	g m^{-2}	plants m^{-2}	w/w %[6]	Foremost	Lethbridge	Warner	seeds m^{-2}	%
2	97.1 (2.7)	6.1 (1.6)	325 (76)	1.06 (0.10)	0.04 (0.34)	0.002 (0.05)	1.075 (7.6)	0 (0.3)	0.108 (0.7)	0.14 (4)	6.2 (11.1)
3	97.2 (2.7)	6.1 (1.6)	321 (76)	1.02 (0.10)	0.09 (0.34)	0.002 (0.05)	0.614 (7.7)	0 (0.3)	0.005 (0.7)	0.22 (4)	3.4 (11.1)
11	93.2 (2.7)	6.1 (1.6)	322 (76)	1.02 (0.10)	0.13 (0.34)	0.002 (0.05)	0.732 (7.6)	0 (0.3)	0.058 (0.7)	0.03 (4)	1.9 (11.1)
Untreated control	0.2 (2.7)	5.8 (1.6)	309 (76)	1.56 (0.10)	2.04 (0.34)	0.105 (0.05)	37.391 (7.6)	0.818 (0.3)	2.217 (0.7)	8.14 (4)	29.3 (11.1)
Contrast	**<0.00005**	NS	NS	**0.0002**	**0.0003**	NS	**<0.00005**	**0.0113**	**<0.00005**	NS	**0.0297**
Sample size	286	286	284	236	190	190	48	48	94	94	95

[1]Values are lsmeans and standard errors from the mixed model ANOVA for separate sites and years. Values below each treatment and untreated control are significance from one tailed estimates. The contrasts were a comparison of the herbicide treatment with the untreated control. NS indicates the means are not significantly different ($P > 0.05$).

[2]Percent control was a visual rating of safflower plants in treated plots relative to the untreated controls. Control of volunteer safflower was evaluated visually six weeks after herbicide treatment (WAT) on a scale of 0% (no control) to 100% (complete control relative to untreated control).

[3]Ellerslie 2004 was excluded from the ANOVA with these variables because no safflower plants or seeds were recovered from any of the harvested barley.

[4]Ellerslie 2005 was excluded from the ANOVA with these variables because no safflower seeds were recovered from any of the harvested barley.

[5]Lethbridge and Warner in 2005 were excluded from the ANOVA with these variables because no viable safflower seeds were recovered from any of the harvested barley.

[6]Percent safflower seed admixture was calculated as a ratio of safflower seed admixed and harvested barley.

Table 6-3. Production response of the four canola systems and transgenic safflower volunteers to herbicide treatments at Ellerslie, Foremost, Lethbridge and Warner in 2004 and 2005.[1]

Canola system	Treatment	Visual control ratings[2]	Canola		Safflower[3]		Safflower admixture[3,4]			
			Yield	Biomass	Biomass	Density	w/w[5]	Total	Viable	
		%	kg ha^{-1}	g m^{-2}	g m^{-2}	plants m^{-2}	%	seeds m^{-2}	seeds m^{-2}	%
Liberty Link	Glufosinate	0.0	2.7 (1.0)	313 (91)	85 (77)	20.7 (8.6)	1.02 (0.61)	26 (21.7)	8.6 (8.0)	21.9 (14.4)
	Untreated control	0.0	2.8 (1.0)	305 (91)	96 (78)	22.5 (8.5)	0.71 (0.49)	27 (21.8)	9.3 (8.1)	25.2 (14.2)
	Contrast	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Sample size	45	45	45	37	37	29	29	29	29
Roundup Ready	Glyphosate	99.545 (0.3)	2.3 (0.9)	289 (97)	8 (35)	1.8 (2.3)	0.03 (0.02)	2 (1.1)	0.2 (3.5)	11.6 (9.5)
	Untreated control	0.002 (0.3)	2.1 (0.9)	250 (101)	86 (41)	17.4 (3.1)	1.06 (0.29)	49 (10.5)	10.4 (4.0)	23.6 (9.0)
	Contrast	**<0.0005**	NS	NS	**0.01695**	**0.00525**	**0.0355**	**0.02245**	NS	NS
	Sample size	46	46	46	38	38	30	30	30	30
Clearfield	Imazethapyr /imazamox	67.009 (15.9)	2.6 (1.0)	319 (126)	7 (7)	3.4 (2.8)	0.12 (0.73)	5 (22.2)	0.4 (6.4)	6.4 (4.5)
	Untreated control	0.442 (15.9)	2.4 (1.0)	316 (122)	54 (13)	8.3 (2.8)	1.59 (0.75)	61 (23.3)	11.2 (6.7)	12.3 (4.2)
	Contrast	**0.0022**	**0.03**	NS	**0.0155**	**0.04025**	NS	NS	NS	NS
	Sample size	46	46	46	38	38	30	30	30	30
Conventional	Sethoxydim /ethametsulfuron	27.503 (15.0)	2.5 (0.9)	274 (68)	37 (27)	7.5 (2.6)	1.45 (1.00)	67 (29.5)	7.3 (4.3)	10.6 (3.0)
	Untreated control	0.674 (15.0)	2.5 (0.9)	259 (66)	40 (27)	7.3 (2.4)	1.35 (0.99)	65 (29.8)	7.5 (4.3)	9.6 (2.6)
	Contrast	**0.0351**	NS	NS	NS	NS	NS	NS	NS	NS
	Sample size	46	46	46	38	38	30	30	30	30

[1]Values are lsmeans and standard errors from the mixed model ANOVA for separate sites and years. Values below each treatment and untreated control are significance from one tailed estimates. Dashes indicate the model did not converge and mean separations could not be determined due to zero values or lack of variance. The contrasts were a comparison of the herbicide treatment with the untreated control. NS indicates the means are not significantly different ($P > 0.05$).

[2]Percent control was a visual rating of safflower plants in treated plots relative to the untreated controls. Control of volunteer safflower was evaluated visually two, four and six weeks after herbicide treatment (WAT) on a scale of 0% (no control) to 100% (complete control relative to untreated control). On some occasions complete control of safflower in all treated plots provided no variance, ANOVA could not be conducted and raw means (SE) are report.

[3]Ellerslie 2004 was excluded from the ANOVA with these variables because no safflower plants or seeds were recovered from any of the harvested canola.

[4]Ellerslie 2005 was excluded from the ANOVA with these variables because no safflower seeds were recovered from any of the harvested canola.

[5]Percent safflower seed admixture was calculated as a ratio of safflower seed admixed and harvested canola weight (w/w%). The percentage of viable safflower admixture was calculated as the ratio of viable safflower seeds and total safflower seeds admixed with harvested canola.

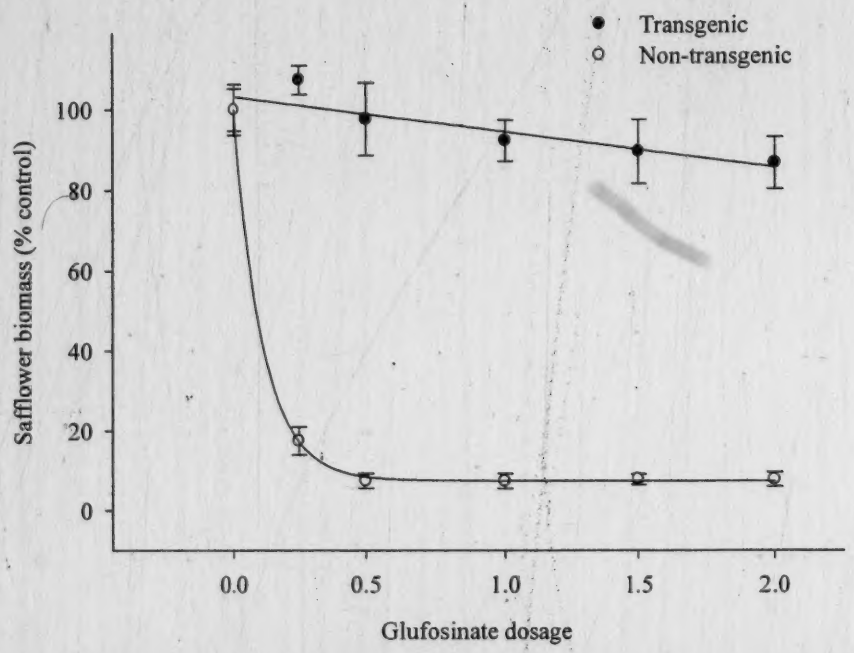

Figure 6-1. Estimated dose-response curves of transgenic and non-transgenic safflower to glufosinate. Circles are means and bars are one standard error. Recommended rate for glufosinate from (Ali, 2003) was used for the 1X dosage and the other doses were relative to it (Tab. 1). Line is the regression line with the optimal model, which was linear and exponential decay for non-transgenic and transgenic safflower, respectively.Sample size for transgenic and non-transgenic safflower was 49 and 53, respectively.

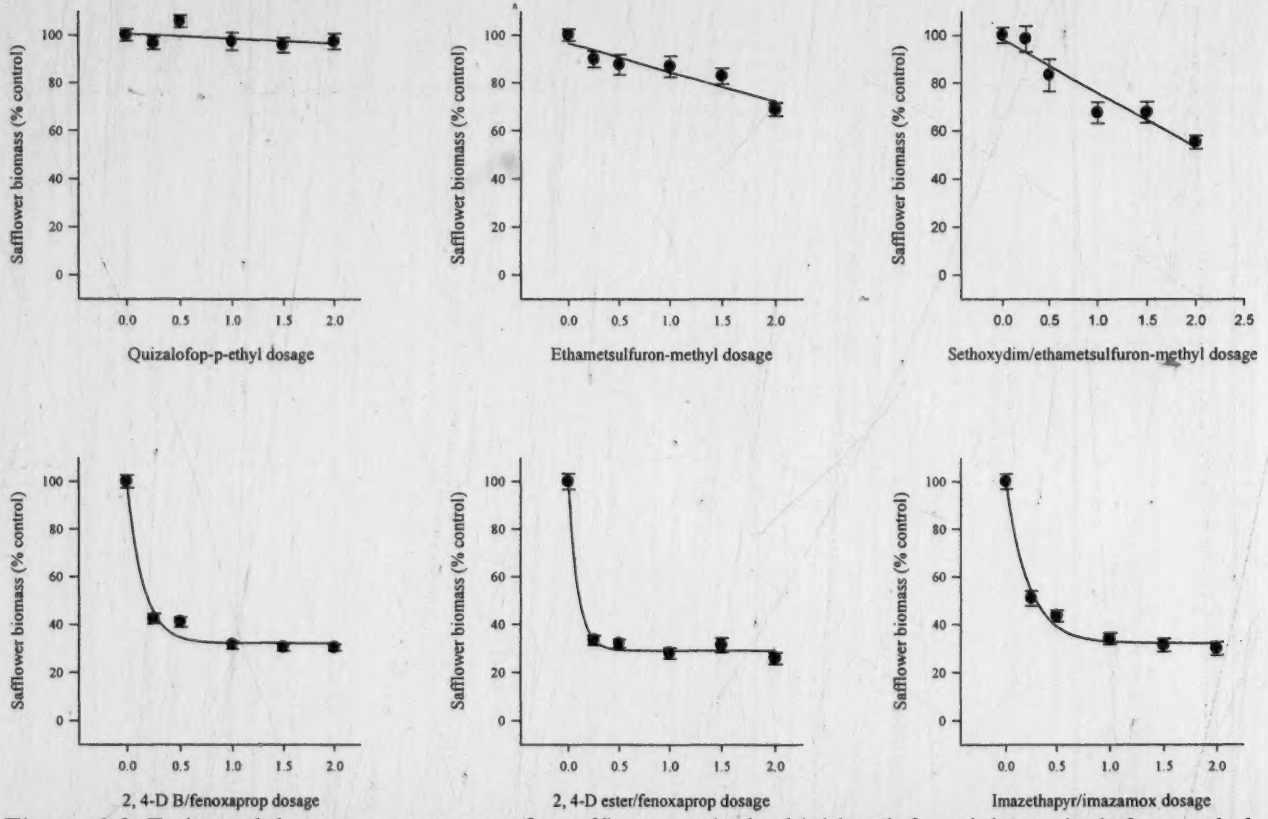

Figure 6-2. Estimated dose-response curves for safflower to the herbicides (left to right): quizalofop-p-ethyl, ethametsulfuron methyl, sethoxydim/ethametsulfuron methyl, 2, 4-D B/fenoxaprop, 2, 4-D ester/fenoxaprop and imazethapyr/imazamox with samples sizes of 144, 108, 107, 141, 108 and 108, respectively. Dose response of transgenic and non-transgenic safflower was not significantly different when these herbicides were applied and data was combined. Recommended rate for each herbicide from (Ali, 2003) was used as the 1X dosage, and the other doses were relative to it (Tab. 1). Circles are means and bars are one standard error. Lines are regression lines with the optimal model which was either linear or exponential decay.

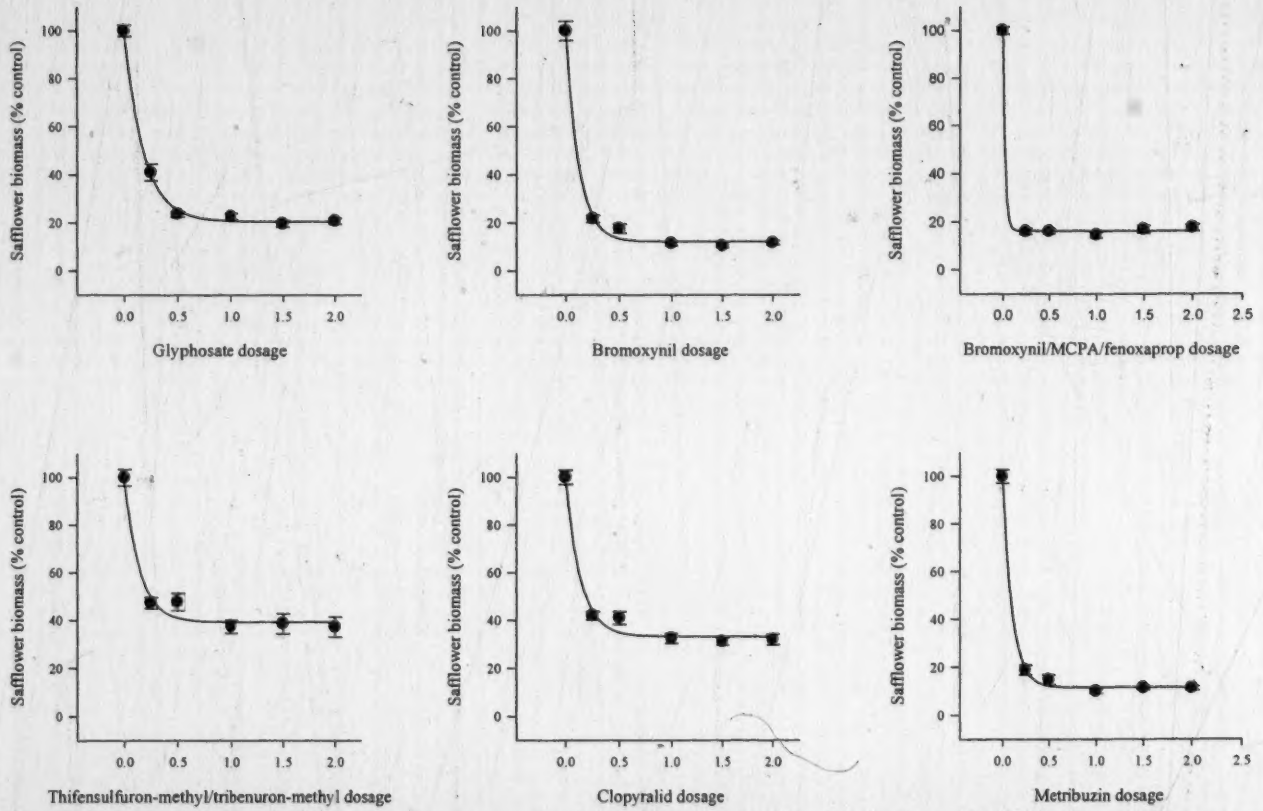

Figure 6-3. Estimated dose-response curves of safflower to the herbicides (left to right): glyphosate, bromoxynil, bromoxynil/MCPA/fenoxaprop, thifensulfuron methyl/tribenuron methyl/fenoxaprop, clopyralid, and metribuzin with samples sizes of 108, 105, 107, 108, 107 and 107, respectively. Dose response of transgenic and non-transgenic safflower was not significantly different when these herbicides were applied and data was combined. Recommended rate for each herbicide from (Ali, 2003) was used as the 1X dosage and the other doses were relative to it (Tab. 1). Circles are means and bars are one standard error. Lines are regression lines with the optimal model, which was either linear or exponential decay.

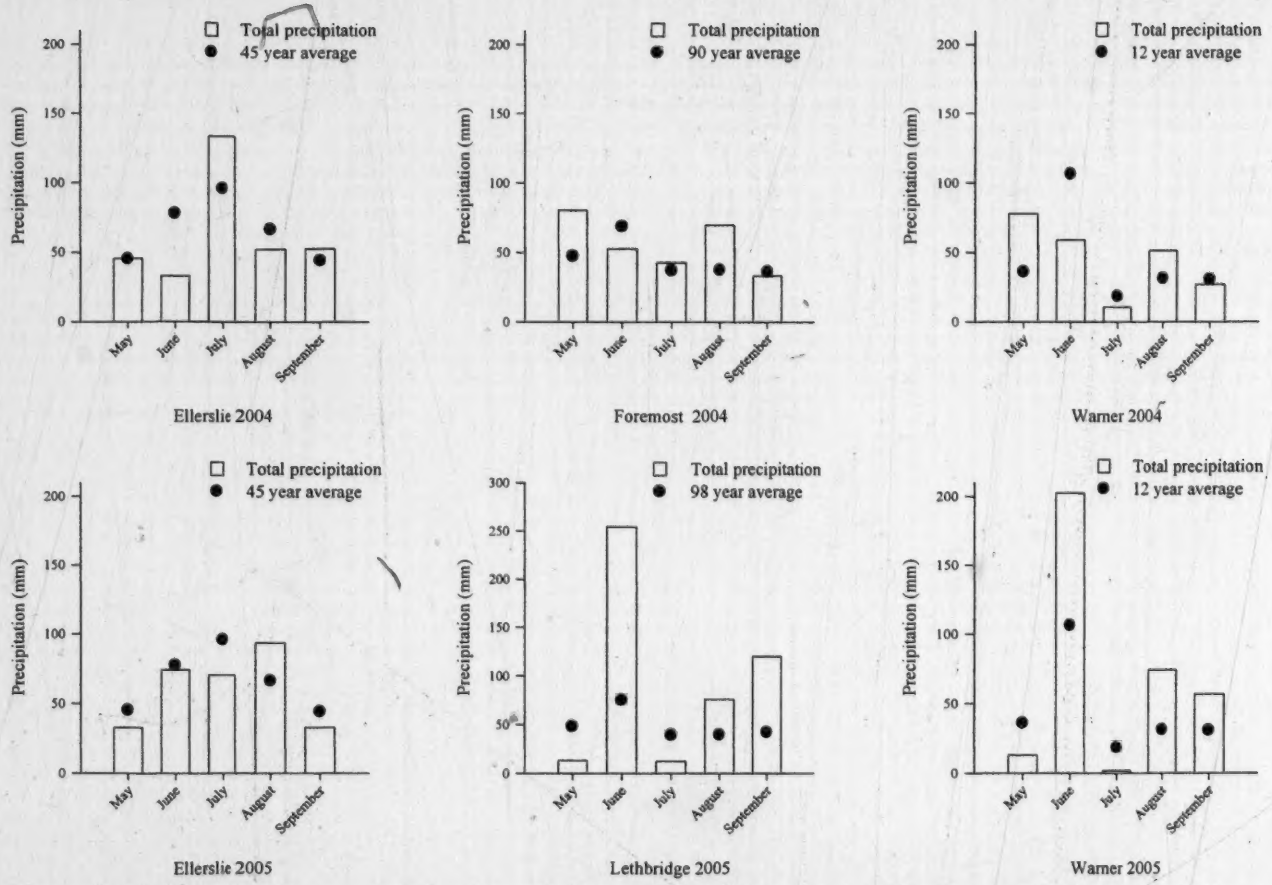

Figure 6-4. Mean monthly precipitation from the time of planting and harvesting the experiments, at all sites and years. The averages are long-term mean monthly precipitation over the time period indicated dating back from 2005.

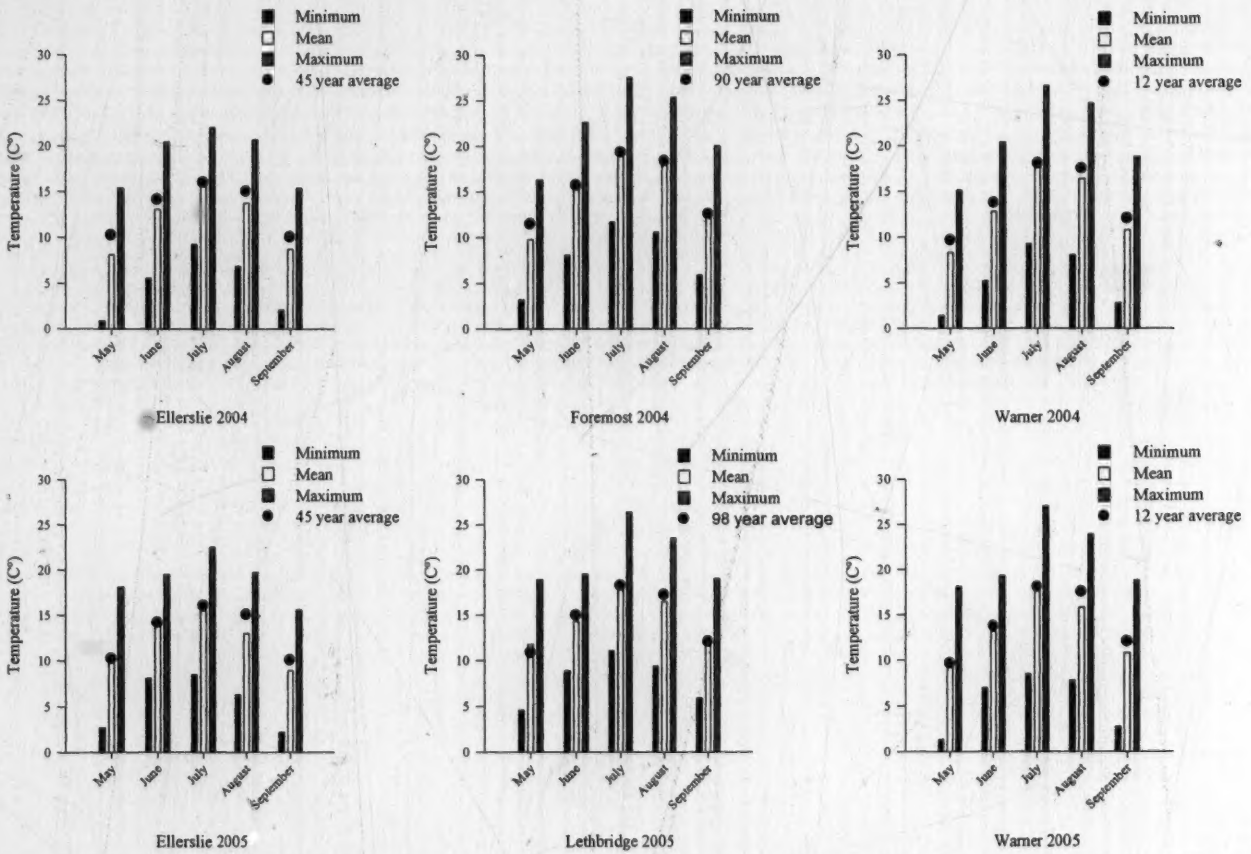

Figure 6-5. Mean monthly temperatures from the time of planting and harvesting the experiments, at all sites and years. The averages are long-term mean monthly temperatures over the time period indicated dating back from 2005.

Appendix

Table 6-A1. Dose-response of transgenic and non-transgenic safflower biomass to several herbicides under greenhouse conditions.

Values presented are means (SE) for estimates from the regression analysis.

Herbicide	Genotype	Intercept[1] *a* or *c*	Slope/rate of decline[2] *m* or *b*	Asymptote *d*	Variance *s*	DF	ED_{50}[3] g ai ha^{-1}	Equation[4]
Quizalofop-p-ethyl	Both	100.7 (2.1)	-2.0 (1.7)	na	217.3 (25.6)	144	>2X[5]	1
Ethametsulfuron-methyl	Both	98.0 (2.4)	-12.2 (2.0)	na	218.4 (29.7)	108	>2X[5]	1
Sethoxydim/ethametsulfuron-methyl	Both	100.7 (3.3)	-22.6 (2.8)	na	395.3 (54.0)	107	>2X[5]	1
Glufosinate	PNT	104.1 (4.5)	-8.7 (3.8)	na	338.9 (68.5)	49	>2X[5]	1
	WT	226.6 (26.9)	8.9 (1.2)	7.4 (1.4)	61.6 (12.0)	53	0.18 (0.01)	2
2, 4-D B	Both	132.2 (11.0)	6.7 (0.8)	32.2 (1.1)	95.1 (11.3)	141	0.20 (0.01)	2
Bromoxynil	Both	204.2 (18.9)	8.4 (0.9)	12.1 (1.0)	68.1 (9.4)	105	0.18 (0.01)	2
Glyphosate	Both	139.2 (6.9)	5.6 (0.4)	20.5 (1.1)	69.8 (9.5)	108	0.22 (0.01)	2
Clopyralid	Both	139.0 (15.3)	7.3 (1.1)	33.2 (1.3)	92.4 (12.6)	107	0.19 (0.01)	2
Imazethapyr/imazamox	Both	106.0 (7.7)	4.5 (0.6)	32.1 (1.7)	134.7 (18.3)	108	0.25 (0.02)	2
2, 4-D Ester /fenoxaprop	Both	212.3 (53.8)	11.0 (2.6)	29.1 (1.3)	110.1 (15.0)	108	0.16 (0.01)	2
Bromoxynil/ MCPA/ fenoxaprop	Both	4804.5 (12709.0)	40.5 (26.4)	16.2 (0.6)	31.3 (4.3)	107	0.12 (0.01)	2
Thifensulfuron-methyl /tribenuron-methyl/fenoxaprop	Both	122.6 (21.7)	7.1 (1.7)	39.3 (2.0)	215.9 (29.4)	108	0.20 (0.02)	2
Metribuzin	Both	235.4 (24.6)	9.8 (1.0)	11.5 (0.9)	53.4 (7.3)	107	0.17 (0.01)	2

[1]Intercept for linear and non-linear equations were *a* and *c*, respectively.
[2]Slope and rate of decline for linear and non-linear equations were *m* and *b*, respectively.
[3]The effective herbicide dose to reduce safflower biomass by 50% relative to the water control.
[4]Optimal equations were chosen by comparison of the data fit to a linear (L) and non-linear model (NL).
[5]ED_{50} values were larger than the doses included in the study.

Table 6-A2. Percent control of volunteer safflower in barley trials 2 and 4 weeks after treatment with herbicides in Ellerlie, Foremost, Lethbridge and Warner in 2004 and 2005.[1]

Herbicide treatment	Visual ratings				
	2 WAT				4 WAT
	%				
	Site				
	Ellerslie	Foremost	Lethbridge	Warner	
2	89.44 (2.4)	85.00 (2.9)	98.33 (2.7)	94.36 (2.4)	94.7 (2.7)
3	89.75 (2.4)	71.30 (3.0)	96.92 (2.7)	79.96 (2.4)	90.8 (2.7)
11	94.52 (2.4)	88.33 (2.9)	80.17 (2.7)	80.84 (2.5)	89.5 (2.7)
Untreated control	0.03 (2.4)	0.00 (2.9)	0.00 (2.7)	0.04 (2.4)	0.0 (2.7)
Contrast	<0.00005	<0.00005	<0.00005	<0.00005	<0.00005
Sample size	96	47	48	95	286

[1]Percent control was a visual rating of safflower plants in treated plots relative to the untreated controls. Control of volunteer safflower was evaluated visually two, four and six weeks after herbicide treatment (WAT) on a scale of 0% (no control) to 100% (complete control relative to untreated control).

Table 6-A3. Results of soil tests for the sites used for the admixture of safflower in barley and canola. Estimated available nutrients for plant growth in top 61 cm of soil for nitrate (N) and sulphate (S) and in the top 15.2 cm of soil for phosphorous (P) and potassium (K).

Year	Site	Crop	OM[1] %	EC[2] dS m^{-1}	pH	N[3]	P Kg ha^{-1}	K	S
2004	Warner	Barley	3.0	0.51	7.0	34	99	>1350	15
	Foremost	Barley	2.1	0.29	7.6	13	49	953	33
	Ellerslie	Barley	10.2	0.39	6.1	43	25	485	40
	Warner	Canola	3.0	0.62	7.8	24	22	853	6
	Foremost	Canola	2.2	0.72	7.0	43	24	754	8
	Ellerslie	Canola	9.2	0.56	5.9	100	111	854	24
2005	Warner	Barley	2.9	0.73	7.2	17	37	779	6
	Lethbridge	Barley	3.0	0.82	7.7	73	49	895	>45
	Ellerslie	Barley	8.0	0.40	6.5	17	22	287	>45
	Warner	Canola	2.5	0.44	7.7	6	20	670	3
	Lethbridge	Canola	4.0	0.86	7.8	54	>135	>1350	>45
	Ellerslie	Canola	11.5	0.35	6.3	35	116	802	11

[1]Organic matter
[2]Electrical conductivity
[3]Nitrate-N method

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Chapter 7: Overview of plant molecular farming with safflower as a platform

Plant molecular farming may provide benefits to society and industry but poses different risks than traditional agriculture to the environment and food/feed system. Thus, it is likely that PMF will require confinement and production channeling measures more stringent than currently employed for other agricultural operations. This research provides a strong background on the environmental biosafety of PMF with safflower as a platform. Several routes of potential transgene movement or persistence in the environment were identified and quantified. Additionally, research results will contribute to risk mitigation strategies and provide information on commodity market implication of PMF production.

It is unlikely that transgenes will be introgressed into wild/weedy relatives if transgenic safflower is grown in some regions of the US and Canada as reviewed in Chapter 2. Four wild/weedy safflower relatives have been introduced into the New World. Of these, only *Carthamus oxyacanthus* and *C. creticus* are cross-compatible with cultivated safflower. Geographic locations where these two species have been reported were identified and should be avoided for PMF with safflower or appropriate isolation distances established from diligent surveys of fields intended for production and surrounding areas.

The influence of domestication traits on the ferality of safflower and the potential for wild/weedy relatives to act as reservoirs for transgenes from PMF safflower were evaluated in Chapter 3. Domestication traits have been selected in cultivated safflower

and include decreased shattering, seed dormancy, pappus length, and duration of the rosette stage. It is likely that these traits would limit safflower persistence within and outside of the agroecosystem. The introgression of transgenes from safflower to one of its cross-compatible wild relatives would be reduced by the linkage of the transgene with genes for domestication. However, the majority of cultivated safflower domestication traits are recessive and in the F_1 hybrids would likely be masked by wild/weedy characteristics. Thus, PMF production with safflower should be avoided where the cross-compatible *C. oxyacanthus* and *C. creticus* are known to occur.

Short distance outcrossing from small plots of transgenic to non-transgenic safflower of the same variety and planted at the same time, was evaluated in Chapter 4. Safflower outcrossing declined rapidly with distance from the pollen source. Outcrossing was 2% within the first few meters of the pollen source but was below 0.05% at distances of 50 to 100 meters. While these experiments were limited by scale permitted by confined release conditions, they can be compared to outcrossing in other crops. The use of a trap crop and isolation distance could be used to mitigate pollen flow from transgenic safflower intended for PMF to commodity safflower.

The loss of safflower seeds at harvest, their persistence in soil at different depths and safflower volunteerism in commercial fields in follow years was reported in Chapter 5 and summarized here in Table 7-3 and Figure 7-1. Losses of viable safflower seeds at the time of harvest were highly variable among fields and ranged from 81 to 518 seeds m^{-2}. Burial of safflower seeds hastened their decline and their viability was reduced by over 50% from fall (harvest September 1) to early spring (follow year May 1). Aside from burial depth the number of viable seeds lost at harvest increases the number of seeds that

will remain viable in the seed bank over time (Tab. 7-3). Safflower volunteer plant densities in commercial fields in follow years were low (Fig. 7-1). Safflower volunteers responded to the stress of competition and perhaps herbicides by reduction in several growth characteristics and rapid maturation. Safflower volunteers did not often survive in wheat and barley and few plants set viable seed. Safflower volunteers in commercial fields under chemical fallow management did not survive to produce flowers or viable seed in any of the fields surveyed. Safflower persistence in the agroecosystem could be reduced by limiting seed losses at harvest, tillage to bury seeds and the use of chemical fallow in follow years to prevent volunteer survival and seed set.

Safflower volunteer survival in the follow crops canola and barley with different in-crop herbicides, admixture of volunteer safflower seeds with harvested canola and barley seed and the efficacy of herbicides with various modes of action to control safflower were reported in Chapter 6 and summarized here in Figure 7-2. Transgenic safflower volunteer survival and fecundity can be reduced by some herbicides and/or competitive crops in follow years. Safflower is a poor competitor in a cereal crop, even in the absence of herbicides, and when common herbicides are applied the likelihood of successful seed set and admixture were reduced. Transgenic safflower can reach reproductive maturity in canola in the absence of herbicides, and some herbicides do not provide satisfactory control of safflower volunteers under some conditions. Several herbicides were found to reduce safflower biomass, but some were more effective than others. In the absence of a threshold, herbicidal or cultural control is unlikely to be sufficient in the first year following PMF production with safflower.

These studies have quantified gene flow via pollen and seed from PMF production with transgenic safflower and provide insights into potential mitigation procedures to limit gene flow. These results can be used to reduce the risk of gene flow from transgenic safflower to the environment and food/feed system and could be used to developed best management practices (BMP). The stringency of the mitigation procedures would be dependent on the acceptable thresholds for adventitious present in commodity products and presence of safflower in the environment. A short summary of potential routes of gene flow from transgenic safflower and BMP to mitigate risks of gene flow for production of transgenic safflower are provided in Tables 7-1 and 7-2.

Table 7-1. A brief overview of some best management practices to limit gene flow from transgenic safflower production to the environment and food/feed system during specific operations.

Crop	Operation	Gene flow route	Concern	Mitigation
PMF safflower	Pre-planting	Pollen	Wild/weedy relatives	Choose sites where wild/weedy relatives do not occur
	Seed treatment	Seed	Seed loss due to pathogens	Chemical treatment of seeds prior to planting
	Planting	Seed	Seed movement off site	Containment of seed and clean seeding equipment after seeding
	Crop production	Seed	Seed loss due to pathogens	In-crop fungicide application (minor use required)[1]
		Pollen	Commodity safflower	Isolation distance from commodity safflower
		Pollen	Commodity safflower	Non-transgenic safflower planted in perimeter of field
		Pollen and seed	Off site movement	Fallow or non-food/feed green manor grown in perimeter
	Harvesting	Seed	Seed losses	Limit seed losses by combine settings and perhaps chaff wagon
	Post-harvest	Seed	Seed movement with residue	Tillage to accelerator loss of viable seed in soil
	Tillage	Seed	Seed movement off site	Clean equipment prior to movement off field
Follow crop	Pre-planting	Seed and pollen	Safflower seed and volunteers	Crop choices include a cereal or chemical fallow
		Seed and pollen	Safflower seed and volunteers	Herbicide(s) to control safflower volunteers
	In-crop[2]	Seed and pollen	Safflower seed and volunteers	Herbicide(s) to control safflower volunteers
	Pre-harvesting	Seed	Admixture with harvested seed	Scouting fields and remove safflower volunteers

[1]No fungicides have been registered for use on safflower in Canada.
[2]Chemical fallow is preferred over a follow crop in the first year after transgenic safflower production for PMF.

Table 7-2. A brief overview of environmental concerns of PMF with a transgenic safflower and some procedures to mitigate pollen and seed mediated gene flow throughout commercial open field production.

Operation	Route	Concern
Training personnel	pollen and seed	Human error
Protocols (standard operating procedures)	pollen and seed	Human error
Scouting fields	pollen and seed	Volunteers flower and produce pollen and seed
Managing volunteers	pollen and seed	Volunteers inside or outside the production area
Cleaning equipment	Seed	Seed on equipment tires and working parts moved offsite

Table 7-3. The percenate of viable safflower seeds remaining over time at three burial depths. The rate of safflower seed viability decline is based on the lowest rates, which were observed at Ellerslie in 2002 (Chap. 5).

	Burial depth (cm)		
Time[1]	0	2	15
Spring 1	42.77000%	10.22000%	6.54600%
Fall 1	21.31000%	1.54600%	0.67960%
Spring 2	5.38300%	0.03700%	0.00770%
Fall 2	2.68200%	0.00560%	0.00080%

[1]Spring and fall are May and September, respectively.

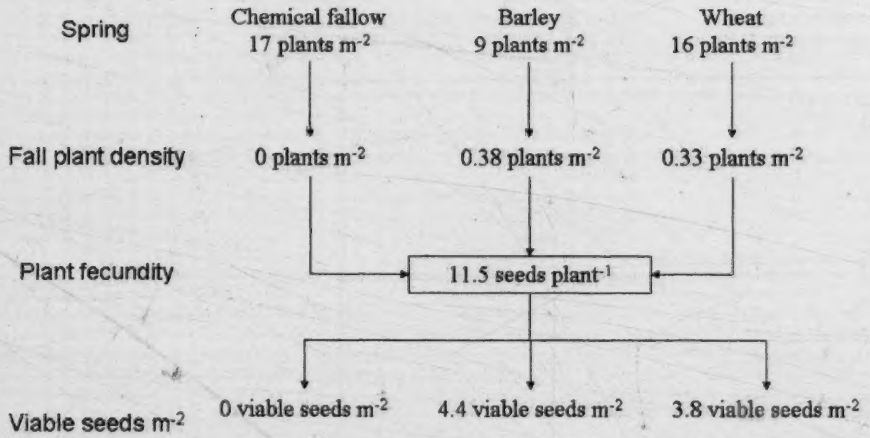

Figure 7-1. Volunteer safflower survival, potential for safflower seed admixture with harvested follow crops and seed bank recharge. Safflower density in spring and fall were the highest mean values found in fields under these management schemes one year after commercial safflower production (Chap. 5; Tab. 5-4). They are the sum of the mean and two standard errors. The fecundity (seeds plant^{-1}) was the sum of the highest mean value and two standard errors for volunteers in commercial fields (Chap. 5; Tab. 5-5). The number of viable seeds available for admixture at harvest and/or recharging the seed bank was estimated by multiplication of the estimated individual fecundity and safflower plant density in fall.

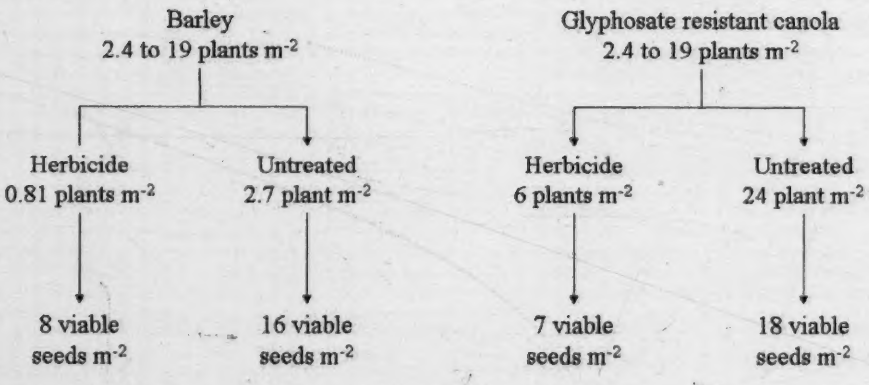

Figure 7-2. A schematic diagram outlining a worst case scenario for safflower volunteer seed admixture in harvested grain from the barley and canola trials presented in Chapter 6. Initial safflower plant densities are minimum and maximum mean emergence values from the admixture trials (Chap. 6; data not shown). Safflower densities and viable admixed seeds are the sum of the highest mean and two standard errors found in the admixture trials (Chap. 6; Tabs. 6-2 and 6-3).

Curriculum Vitae

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Biographical Information

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Education

Ph.D. candidate. University of Alberta, Department of Agriculture Food and Nutrition, Edmonton, Alberta, Canada (Sept. 2003 –), Biological Safety of biopharming plant-made pharmaceuticals.

Supervisor: Linda M. Hall

M.Sc. University of Alberta, Department of Biological Sciences, Edmonton, Alberta, Canada (Sept. 1999 – Jan. 2002), Systematics and Evolution. Molecular systematics of Asparagales.

Supervisor: Sean W. Graham

B.Sc. Zoology with Distinction (Sept. 1994 – Apr. 1999), University of Lethbridge, Lethbridge, Alberta, Canada.

Employment

Research assistant: Alberta Agriculture and Food, Edmonton, AB. (Oct. 2004 – current)

Supervisor: Linda M. Hall

Teaching assistant: University of Alberta, Edmonton, AB. Instruction and preparation of undergraduate labs for a second year introductory plant science course. (Sept. 2003 – Dec. 2003; Sept. 05 – Dec. 05)

Supervisor: Gail Rankin

Technician: Alberta Agriculture and Rural Development, Edmonton, AB. Field experiments related to the biological safety of growing transgenic plants and several studies related to herbicides, crops and the environment. (Jan.2002 – Sept 2003).

Supervisor: Linda M. Hall

Teaching assistant: University of Alberta, Edmonton, AB. Teaching assistant and preparation for undergraduate a first year cellular biology course and a second year botany course. (Sept. 1999 – Apr. 2002)
Supervisors: Kim Christopher and Ruth Stockey

Technician: A large number (ca. 1000) anaerobic bacterial strains were isolated from the rumen of various domestic and wild ruminant animals and screened for their ability to produce bacteriocins (proteinaceous antimicrobials). (Dec. 1997 – Aug. 1999).
Supervisors: Ron M. Teather and Mark F. Whitford

Co-op work term 2: Several cold inducible genes and their promoters were isolated from potato plants using molecular techniques (Jun. 1997 – Dec. 1997)
Supervisor: Jin Hao Liu

Co-op work term 1: RAPD-PCR using ssDNA were used to screen several wheat lines for resistance to various plant pathogens (Jan. 1997 – Apr. 1997)
Supervisor: Andrea Laroche

Safety Courses

Standard first AID / CPR – Training. University of Alberta, Occupational and Health and Safety. Edmonton, Alberta, Canada (Sept. 2002).

Radiation safety course. University of Alberta, Occupational and Health and Safety. Edmonton, Alberta, Canada (Sept. 2001).

Academic Awards, Scholarships and grants (Canadian funds listed)

Dow Agrosciences and Syngenta Travel Award	2007	$1,000
Queen Elizabeth II Graduate Fellowship	2005	$10,500
Provost Doctoral Entrance Award, University of Alberta	2004	$4,000
Iris Society of America Scholarship	2001-2002	$2,800
Challenge Grant in Biodiversity, University of Alberta	2000-2001	$6,800
Deep Green Student Travel Grant	2000	$500
Academic Award of Achievement, Red Deer College	1995	$500

Teaching Experience

Teaching assistant for Plant Science 221 Laboratory, Introduction to plant science (three terms, 2004-2005), Department of Agriculture Food and Nutrition Sciences University of Alberta

Teaching assistant for Botany 210 Laboratory, The plant kingdom: vascular plants (one term, 2002), Department of Biological Sciences University of Alberta.

Teaching assistant for Biology 107 Laboratory, Introduction to Cell Biology Laboratory (five terms, 1999-2002), Department of Biological Sciences University of Alberta.

Refereed Publications

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Invited Presentations

Hall, L. M. and M. A. McPherson. 2007. Environmental Biosafety of Plants for Molecular Farming. Pharming the Future. A citizen's consultation on plant molecular farming. Jan 26 and 27. Genome Canada, National GE3LS (Genomics, Ethics, Environment, Economics, Law and Society) Research Network Calgary. Alberta. Presentation on gene flow, crop choice and plant molecular farming.

McPherson, M. A. 2006. Plant-made pharmaceuticals in safflower. Is this crop a weed? Alberta Weed Advisory Committee. Edmonton, AB.

Hall, L. M. and M. A. McPherson. 2005. Confinement of Plant-Made Pharmaceuticals - Gene Flow via Seed and Volunteers of Safflower (*Carthamus tinctorius*). Proceeding APHIS conference of confinement of plant made pharmaceuticals.

Hall, L, M. and M. A. McPherson. 2004. Confinement of transgenes in seed and volunteer populations. Confinement. Workshop on Confinement of Genetically Engineered Crops During Field Testing. USDA-APHIS. Rosedale Maryland.

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Whitford, M. F., McPherson, M. A. and Teather, R. M. 2000. Identification and purification of bacteriocins from novel rumen bacteria. 100th General meeting of the American Society for Microbiology, Los Angeles, California. (Poster presentation).

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Dexter, J. E., M. A. McPherson, L. M. Hall, A. Snow. 2005. Biosafety Implications of the Introduction of Imidazolinone-Resistant Sunflower (*Helianthus annuus* L.) in Canada. Report commissioned by Canadian Food Inspection Agency.

Hall, L. M., K. Topinka, M. A. McPherson, E. Phillipchuk, and A. G. Good. 2003. Assessing Gene Flow –Plant-Made Pharmaceuticals. Final Report to the IDS New Initiatives Fund.

Professional Affiliations

American Society of Plant Taxonomists (2001-2003).

Canadian Weed Science Society (2004-current).

The Council for Agricultural Science and Technology (2005- current).

International Society of Biosafety Research (2006-current).